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Effects of ovulen-50, diethylnitrosamine and phenobarbital on liver regeneration in female rats

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Abstract. Short term effects of ovulen-50, a combination type oral contraceptive agent and phenobarbital—an established hepatic tumour promoter, were examined in the livers of diethylnitrosamine-initiated and uninitiated female rats. Liver mitotic activity as judged by liver weight, [^{3}H]thymidine incorporation into DNA and levels of DNA, RNA and protein were measured in non-regenerating and regenerating liver. Hepatic γ-glutamyl transpeptidase activity and hepatocyte agglutination with concanavalin A were examined in diethylnitrosamine- and/or phenobarbital-treated rats.

The results indicate that diethylnitrosamine or ovulen-50 individually are mitoinhibitory in regenerating liver. Phenobarbital alone has a slight mitostimulatory effect in non-regenerating liver, but no effect on liver regeneration. Administration of ovulen-50 and phenobarbital to diethylnitrosamine initiated rats mitigated the mitoinhibition during regeneration. Contrary to the earlier observation with ovulen-50, neither phenobarbital nor diethylnitrosamine induced hepatocyte agglutination in the presence of concanavalin A. Like ovulen-50, diethylnitrosamine also increased the level of hepatic γ-glutamyl transpeptidase. Phenobarbital produced only insignificant rise and did not substantially exacerbate the effect diethylnitrosamine.

The data show that though some of the effects of ovulen-50 resemble those of diethylnitrosamine or phenobarbital, the changes observed may not be related to the neoplastic phenomenon since they were not seen in an initiator-promoter combination regimen.

Keywords. Oral contraceptive agent; phenobarbital; diethylnitrosamine; liver regeneration; nucleic acids; γ-glutamyl transpeptidase; concanavalin A; hepatocytes agglutination.

Introduction

An association between the use of estrogens and higher incidence of hepatocellular adenoma and possibly carcinoma has been reported. A number of studies have been conducted to clarify the role of estrogens in neoplasia. In some studies estrogens were found to act as complete carcinogens when fed to animals as estrogen-progestin combination for up to 104 weeks. The results varied with the species, strain and estrogen dose (Schardein *et al.*, 1970). Other studies show that estrogens are not themselves carcinogens, but promote the effects of carcinogens (Yager and Yager, 1980).

There are very few studies wherein the effects of steroidal contraceptives on the liver have been examined from a functional point of view using biochemical parameters. Also most of the earlier studies have employed very high doses of hormones.

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Abbreviations used: OCA, Oral contraceptive agent; pH, partial hepatectomy; GGT, γ-glutamyl transpeptidase; Con A, concanavalin A; DEN, diethylnitrosamine; PB, phenobarbital.

An increased accumulation of fat after carbontetrachloride (CCl_4) injury in the livers of Enovid-treated compared to untreated control female rats has been reported (Joshi and Rao, 1969). However, the recovery of the liver following CCl_4 injury though delayed by Enovid was found to be complete. In another study on female rhesus monkeys, it was observed that the increase in serum enzymes of hepatic origin seen after the administration of aflatoxin a powerful hepatocarcinogen could be prevented by the simultaneous administration of ovulen an oral contraceptive agent (OCA) (Belavady *et al.*, 1973). These results were supported by the work of Mg bodile and Holscher (1976), who found that the pre-administration of 100 μg ovral-28 for 16 days mitigated the acute effects of a single dose of aflatoxin B_1 in rat liver.

Studies of Mukundan *et al.* (1981) show that daily administration of 5 μg ethinylestradiol, 100 μg ethynodiol diacetate in the form of OCA-ovulen-50 to female rats leads to an increase in liver lipids and mitostimulation in a non-regenerating liver, but mitoinhibition in liver regenerating after partial hepatectomy (pH). We have recently shown that after similar treatment, there is a slight increase in the level of the tumour marker enzyme γ-glutamyl transpeptidase (GGT) and increased agglutinability of isolated hepatocytes with concanavalin A (Con A) suggesting certain degree of liver damage and/or transformation in OCA-treated female rats (Annapurna *et al.*, 1987).

Inhibition of cell proliferation and/or DNA synthesis is also brought about by many carcinogens and this is considered to have some significance in the carcinogenic process (Farber, 1976).

The studies reported in this paper were designed to find out if established tumour initiator and promoter compounds like diethylnitrosamine (DEN) and phenobarbital (PB) respectively would behave like OCA with regard to the above cited effects, when administered singly and in combination over a short duration of 7 weeks.

Experimental

Ovulen-50 was obtained from Searle Company, Bombay. DEN from Sigma Chemicals, St. Louis, Missouri, USA, and phenobarbital from Indian Drugs and Pharmaceuticals, Hyderabad. [^{3}H]Thymidine was obtained from Bhabha Atomic Research Centre, Bombay. All other compounds used were of the highest purity available.

Two experiments were carried out. In the first experiment 64 weanling female rats of Wistar/NIN strain were divided into two equal groups. One group received a single intraperitoneal injection of DEN (200 mg/kg body weight) in saline (initiated rats). The other group received the same quantity of saline and served as uninitiated control. Four weeks after this injection, 16 rats from DEN-initiated group and 16 from saline-treated group received either propylene glycol or one-tenth tablet of the OCA-ovulen-50 as propylene glycol suspension for 7 weeks. Three or four rats from each of the four treatment groups—(i) control, (ii) DEN, (iii) OCA, (iv) DEN+OCA-treated were sacrificed at 0, 24, 48 and 72 h after pH. pH was performed by removing two third of the liver under light ether anesthesia (Higgins and Anderson, 1931). One hour before sacrificing, 5 μCi of [^{3}H]thymidine (sp. act. 9·8 μCi/mmol) portion of the non-regenerating liver was used for histopathological studies by nventional methods and 6 μm paraffin sections were stained and examined.

Throughout the experiment, the animals were fed a balanced diet containing 20% ein, 63·8% starch, 10% groundnut oil, salt and vitamin mixture from the time of anling till sacrifice. They were housed in individual screen-bottom cages and intained at 22–25°C in 12 h light-dark cycle. Body weights were recorded at ekly intervals.

The design of the second experiment and parameters measured were similar to first experiment except that in place of OCA, 0·05% PB was administered ough diet. The animals were sacrificed at 0 and 24 h after pH. The 24 h time int was used since maximum effect on liver regeneration with OCA treatment s seen at that time point. In addition to the mitotic activity, levels of GGT and patocyte agglutination with Con A were also examined in non-regenerating livers methods described earlier (Annapurna *et al.*, 1987).

Statistical analysis of the data was done by using analysis of variance with or thout log transformation of the values and Duncan's multiple range test, nsidering data on the effects of treatment and time after partial hepatectomy at rious time points together.

sults

H] Thymidine incorporation and levels of DNA, RNA and protein in non-generating and regenerating liver.

the first experiment the mitostimulatory effects of OCA on non-regenerating liver d the mitoinhibitory effect on regenerating liver reported earlier from our oratory were confirmed (figure 1) (Mukundan *et al.*, 1981; Annapurna *et al.*, 87). DEN alone had similar effects of greater magnitude. Surprisingly, however, en OCA was given to DEN-initiated rats there was no exacerbation. On the ntrary, mitoinhibition after pH seen with each of these agents individually, was mpletely mitigated when they both were administered and the process of eneration was found to be normal in these rats (figure 1).

Results of the second experiment show that PB *per se* tended to increase the [^{3}H]-ymidine incorporation into non-regenerating liver, suggesting stimulation of totic activity. However, this trend was not significant in the liver weight and NA, RNA and protein contents (figure 2). PB had no effect on regeneration of er after partial hepatectomy. When administered to DEN-initiated rats, PB tigated the effect of DEN on non-regenerating as well as regenerating liver (figure 2).

ects on liver GGT activity

EN produced significant rise in liver GGT activity. PB showed a similar but non-nificant trend. The GGT activity was highest in rats which had been initiated th DEN and subsequently treated with PB, but this increase was not significantly ore than DEN-treatment alone (table 1).

epatocyte agglutination with Con A

EN or PB singly or in combination showed no effect on agglutination of

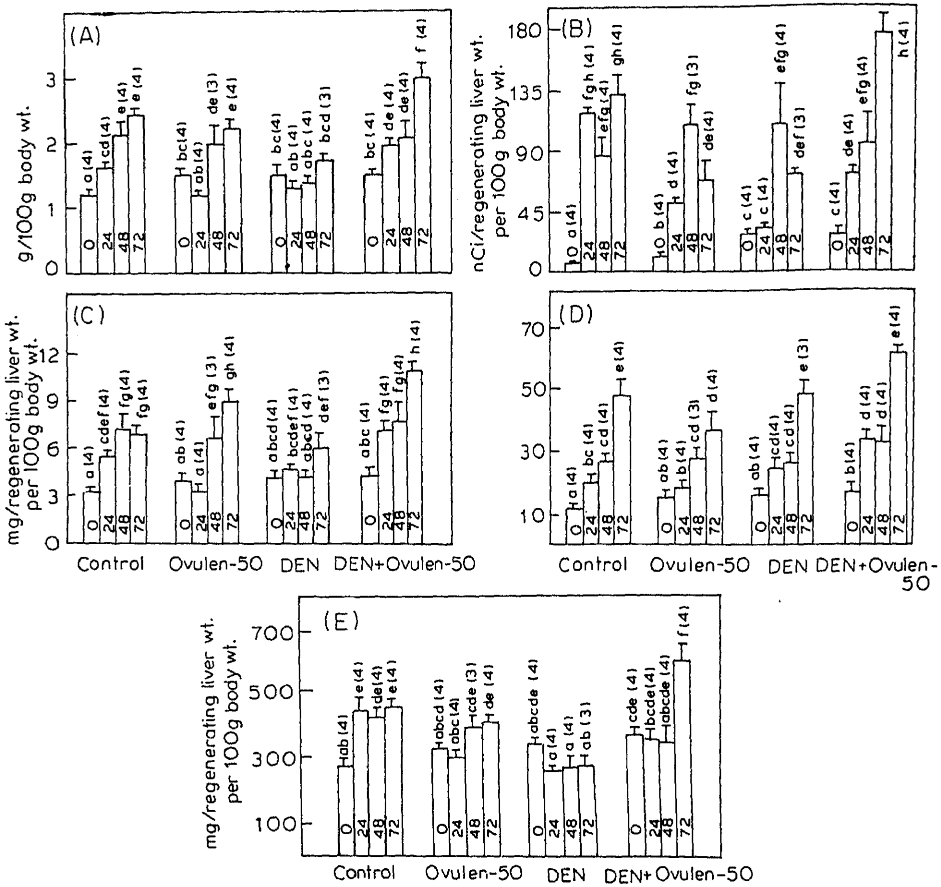

Figure 1. Liver regeneration in DEN and/or ovulen-50 treated female rats. (A), Liver weight; (B), [^{3}H]thymidine incorporation into liver DNA; (C), DNA; (D), RNA; (E), protein.

Results are presented as mean ±SE for groups of 3–4 rats. Statistical analysis of the data was done by using analysis of variance with or without log transformation of the values and Duncans multiple range test, using the data on the effects of all the treatments and time points after partial hepatectomy together. Bars not sharing even one common superscript a, b, c, d, e are significantly different ($P<0{\cdot}05$). 0, 24, 48 and 72 represent time in hours after pH. Numbers in parentheses indicate number of animals used.

Histology

The livers of rats from the OCA or PB-treated groups were not remarkably different from the controls. However, livers of animals given DEN alone or in combination with PB or OCA exhibited a few scattered foci of vacuolated cells.

Discussion

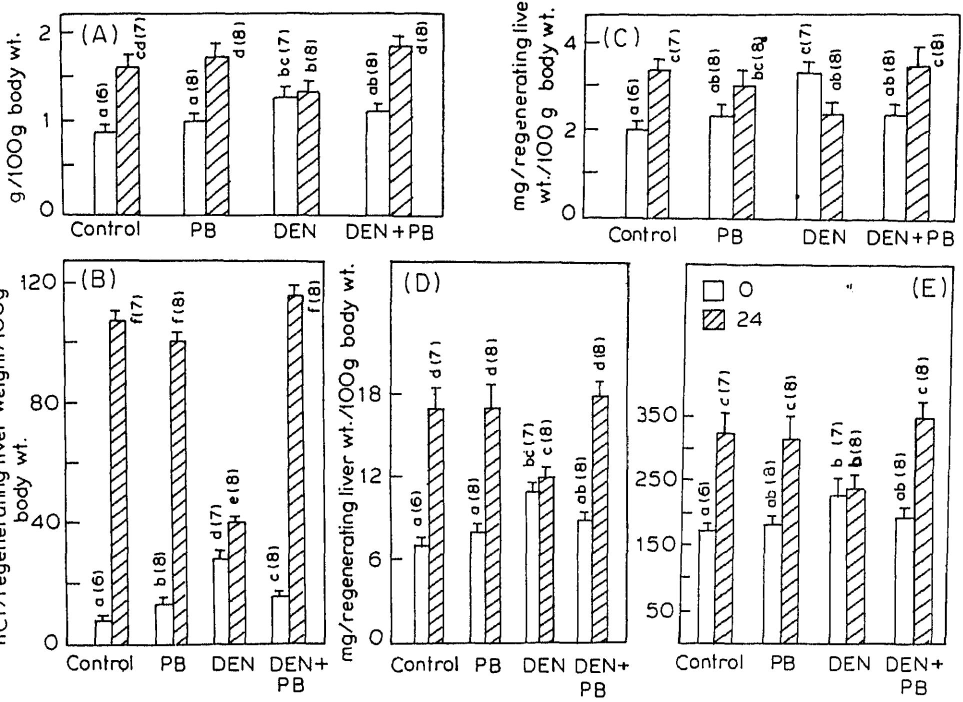

Figure 2. Liver regeneration in DEN and/or PB treated female rats. (A), Liverweight; (B), [^{3}H]thymidine incorporation into liver DNA; (C), DNA; (D), RNA; (E), protein.

Results are presented as ±SE for groups of 7–8 rats. Statistical analysis of the data was done by using analysis of variance with or without log transformation of the values and Duncans multiple range test, using the data on the effects of all the treatments and time points after pH together. Bars not bearing even one common superscript are significantly different ($P < 0{\cdot}05$). 0 and 24 represent time in hours after partial hepatectomy. Numbers in parentheses indicate number of animals used.

Table 1. Effect of PB treatment for 7 weeks on GGT activity in the liver of DEN initiated and uninitiated female rats.

	Treatment			
	Control	PB	DEN	DEN+PB
GGT activity				
Total activity nmol/mt/g liver	$143{\cdot}10 \pm 9{\cdot}902^{a}$ (6)	$161{\cdot}62 \pm 10{\cdot}279^{ab}$ (8)	$187{\cdot}59 \pm 14{\cdot}261^{bc}$ (7)	$222{\cdot}22 \pm 19{\cdot}279^{c}$ (8)
Specific activity nmol/mt/mg protein	$0{\cdot}763 \pm 0{\cdot}043^{a}$ (6)	$0{\cdot}883 \pm 0{\cdot}074^{ab}$ (8)	$1{\cdot}093 \pm 0{\cdot}116^{bc}$ (7)	$1{\cdot}233 \pm 0{\cdot}109^{c}$ (8)

Values are mean ±SE.
Numbers in parentheses indicate the number of animals used.
Data analysed by analysis of variance and Duncans multiple range test

It raised the levels of the tumour marker enzyme GGT in the liver and made hepatocytes susceptible to agglutination by Con A (Annapurna *et al.*, 1987).

The object of the present investigation was to find out if these biochemical and morphological changes can be labelled as early markers of neoplastic phenomenon and thereby ascertain the neoplastic risk of OCA in a short duration study. A two stage initiator-promoter model of hepatic tumourogenesis as described by Wanless and Medline (1982) was used in which either OCA or PB (a known tumour-promoting agent) were administered to female rats which had been initiated with DEN (a known tumour-initiating agent). Suitable control and single-treatment groups were also included for comparison.

The data reported show that the action of OCA on several of the parameters such as mito-stimulation of non-regenerating liver, mito-inhibition of regenerating liver and increase in liver GGT activity (Annapurna *et al.*, 1987) resembles that of DEN, a tumour initiator. Yet the first two effects were not seen in rats treated with DEN-OCA combination or an established initiator promoter combination like DEN and PB.

Hepatocyte agglutination with Con A earlier reported for OCA-treated rats was also not observed with DEN and/or PB treated rats. From these observations it appears that the observed changes may not be related to the neoplastic phenomenon and cannot be used as early markers of tumourogenicity.

Impaired liver regeneration after partial hepatectomy has been observed in conditions such as vitamin A deficiency (Jayaram *et al.*, 1975), some alkaloid treatment (Downing and Peterson, 1968) and alcohol treatment (Lieber, 1981) in rats. These treatments are known to produce liver damage but not hepatic neoplasia.

Membrane changes brought about by OCA as reflected in hepatocyte agglutination with Con A may not have any bearing to the neoplastic process in view of the fact that similar hepatocyte agglutination with Con A treatment is also brought about under some non-carcinogenic conditions like the adaptive changes to diet and drugs (Henriquez *et al.*, 1979; Nicolson *et al.*, 1986). Thus the agglutination noted with OCA-treated rat hepatocytes could be a result of an adaptive change.

The 2-fold rise in GGT activity of OCA treated hepatocytes observed earlier (Annapurna *et al.*, 1987) is of a lesser magnitude than the 15–150-fold increase seen in tumour cells. An increase in hepatic GGT activity of a smaller magnitude has been reported in other conditions of liver damage such as alcoholism and following use of drugs such as phenytoin, aminopyrine, etc. (Goldberg 1980). The slight increase in serum alkaline phosphatase (Annapurna *et al.*, 1987) together with an increase in GGT activity in liver may indicate a small degree of hepatic damage, perhaps mild cholestasis.

It is therefore possible that under the conditions of experimentation, OCA-ovulen-50 is hepatotoxic and not neoplastic in female rats.

The observation that alterations produced by each of these agents DEN, OCA and PB when administered singly were not exaggerated and even abolished when given as initiator-promoter combination regimen is difficult to explain. It may be related to an interplay of drug metabolizing enzymes induced by these agents (Lapis neoplastic is substantiated by the results of a study recently reported by us wherein OCA when administered for a longer period of 60 weeks in an initiator promoter regimen, failed to produce neoplasia (Annapurna *et al.*, 1988).

The rat model to study the effects of OCA used in the present study has been used in several earlier studies involving different types of OCA effects (Ahmed and Bamji, 1976; Mukundan *et al.*, 1981; Annapurna *et al.*, 1987; Vijayalakshmi and Bamji, 1987). Though on the body weight basis the dose of hormones employed is almost 25 times the human dose this cannot be regarded as a very high dose since the effective contraceptive dose of hormones on the basis of body weight is 10 times higher for rats as compared to humans (Banik and Revesz, 1968). Metabolic disposal of these hormones is much faster in rats than in humans (Schwenk *et al.*, 1979).

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Radioimmunoassay of polypeptide hormones using immunochemically coated plastic tubes

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Abstract. A method has been developed for immobilisation of antisera on fresh plastic tubes through an immunochemical bridge. This type of immobilisation has been shown to be more consistent than direct adsorption on plastic. Such immunochemically coated antisera on plastic tube has been used in the development of a noncentrifugation radioimmunoassay. This assay system has been found to be technically as sound as the conventional method.

Keywords. Radioimmunoassay; polypeptide hormones; normal rabbit serum; bovine serum; antisera.

Introduction

Radioimmunoassay (RIA), a technique extensively used in all endocrinological nvestigations, utilizes the double antibody procedure for the separation of bound hormone. This method of separation is time consuming and requires additional eagents. Solid phase RIA's are developed in many laboratories, using antisera (a/s) mmobilised on sephadex, cellulose etc. (Leif Wide, 1981; Chakrabarti *et al.*, 1985). Attempts have been made to improve this system using antibody adsorbed on plastic tube, further eliminating a centrifugation step (Parsons, 1981; Murthy and Moudgal, 1986). In the present study, we have immobilised a/s on locally available plastic tube through an immunochemical bridge and report on the advantages of using such a system in RIA.

Materials and methods

Plastic tubes were purchased locally from Sarad Biochemicals, Bangalore. These were moulded of high density polyethylene. All hormone a/s used in these nvestigations were raised by the conventional procedure in rabbit/monkey. Antisera for human chorionic gonadotropin (hCG) and ovine follicle stimulating hormone (oFSH) were raised in rabbit and monkey respectively. AntiIgG (to rabbit or monkey) was raised in goats. hCG was a gift sample from NIH, USA. All other eagents used were of analytical grade. Buffer used for RIA had 0·05 M phosphate and 0·025 M EDTA in physiological saline, pH 7·4 and had 1% bovine serum (BS) referred as RIA buffer).

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Abbreviations used: RIA, Radioimmunoassay; a/s, antisera; hCG, human chorionic gonadotropin; oFSH, ovine follicle stimulating hormone; BS, bovine serum; PEG, polyethyleneglycol; hLH, human

Preparation of immunochemical bridge

Fresh plastic tubes were coated with 0·5 ml of normal rabbit serum (I) diluted in water (1/1000 diluted), care being taken to see that the solution was pipetted to the bottom of the tube preventing the formation of droplets of the coating solution on the sides of the tube. Following overnight coating, free sites were saturated with in RIA buffer for 1 h. For extension of the bridge the tubes were washed, and 0·6 ml antiIgG (A) was added at high concentration (1/300 in RIA buffer, 4°C) to allow saturation of the IgG bound to the plastic surface with A. At this stage we have IgG-antiIgG (-IA) bridge. Further extension of the bridge, when required, was carried out as above using saturating concentration of IgG and antiIgG to obtain required length of the immunochemical bridge.

Preparation of the bridge starting from A was carried out in a similar fashion. In all cases immunochemical bridges were made in such a way that the end point of the bridge contains antiIgG. These were then stored at 4°C or −20°C until use. Similarly -IA bridges for monkey system was prepared in an identical fashion using normal monkey serum and antiIgG (monkey) in the goat.

Adsorption of a/s on immunochemical bridge

Plastic tubes with immunochemical bridge was washed twice with RIA buffer containing 1% BS, drained on a filter paper for 5–10 min. To these tubes 0·6 ml of a/s (1/10,000 to 1/300,000) diluted in RIA buffer was added. The primary requisite for coating a/s is that the antiIgG used should be against the IgG of the species in which the a/s is raised. Binding of a/s was allowed to proceed at 4°C overnight and the tubes were washed twice with 1 ml RIA buffer. These tubes were then stored at 4°C/−20°C until used.

Quantitation of adsorption of a/s

Antisera at 1/100,000 was added to immunochemically coated tube (600 μl). After the adsorption the quantity of a/s remaining in the supernatant was measured with respect to control a/s, by its binding to ^{125}I-hCG. The quantity of a/s adsorbed was then quantitated by the decrease in the binding of ^{125}I-hCG.

Direct adsorption of a/s on plastic

The adsorption was carried out as described by Murthy and Moudgal (1986). Briefly a/s at 1/10,000 dilution in distilled water was added (0·5 ml) to fresh plastic tubes and allowed to adsorb at room temperature overnight. The a/s was discarded, and RIA buffer (0·6 ml) was added to these tubes to saturate the unsaturated binding sites. After 1 h the solution was discarded and tubes were stored at 4°C until further use.

Binding of ^{125}I-hormones to a/s coated tubes

Antisera coated tubes were washed with RIA buffer, drained and to each tube ml of RIA buffer containing 50,000–100,000 cpm of ^{125}I-hormone was added. e tubes were left on the bench overnight. Next morning the solution was poured ., tube washed once with RIA buffer and counted in a LKB gamma counter.

mpetitive binding assay with a/s coated tubes

tisera coated tubes were incubated with sample or hormone (varying amount in :h tube) and ^{125}I-hormone, all in RIA buffer in a total volume of 0·6 ml as eady described (Murthy and Moudgal, 1986). Incubation was carried out on the ıch for 20 h at room temperature, the tubes drained and washed once with 1 ml A buffer and counted. Conventional competitive binding assays were carried out the standard procedure using double antibody-polyethyleneglycol (PEG) for aration of the bound hormone from the free hormone (Murthy and Moudgal, 37).

Binding assays were analysed by Scatchard method (Scatchard, 1949). In each ılysis correction for the damage of hormone during iodination was applied as cribed (Murthy and Freisen, 1985).

sults

ure 1 shows the optimisation of the antiIgG dilution to be used for the

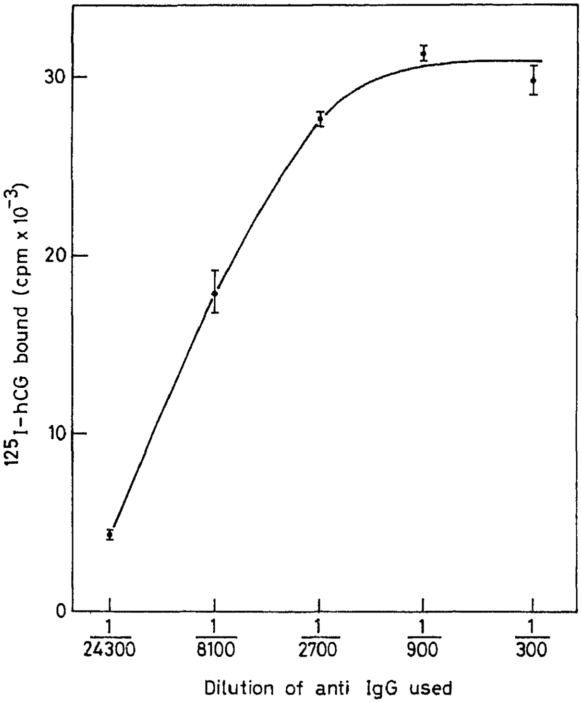

Figure 1. Titration of antiIgG concentration required for saturation of IgG adsorbed on the plastic tube. Plastic tubes were first coated with NRS at 1/1000 dilution in water followed by saturation with antiIgG at varying concentration.

preparation of the immunochemical bridge. It is seen that a dilution of 1/2000 is required to saturate all the IgG bound to the plastic tube. If the saturation needs to be done in shorter time a higher antiIgG concentration (1/100) can be used reducing this period to 2–3 h.

Figure 2 shows the time course of adsorption of a/s by the immunochemical bridge by (i) ^{125}I-hCG binding to the a/s adsorbed tube (curve a) and (ii) by decrease in the binding of ^{125}I-hCG to the unadsorbed a/s from the tube in conventional binding assay (curve b). It is seen clearly that increased a/s adsorption as measured by both methods are nearly parallel indicating that binding of a/s on the solid phase through immunochemical bridge results in uniform retention of activity of the a/s.

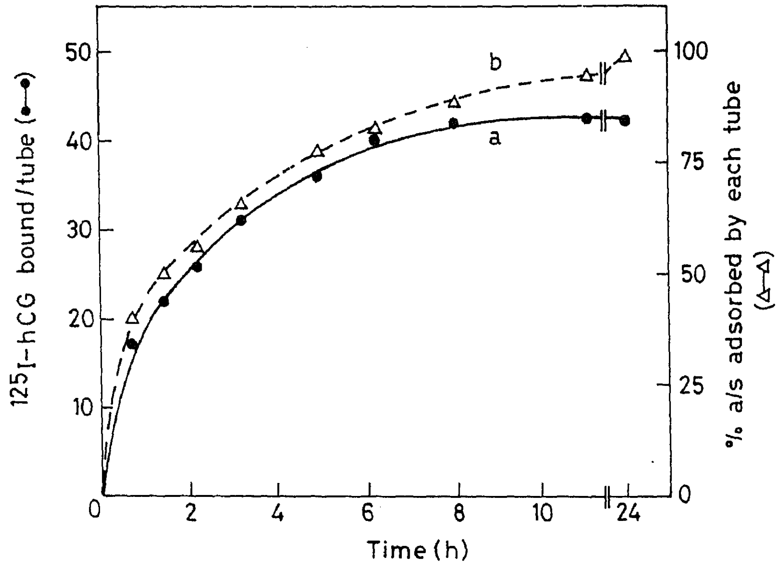

Figure 2. Adsorption of a/s by -IA tubes with time. Curve (a), ^{125}I-hCG bound to a/s coated plastic tube; curve (b), per cent a/s adsorbed by -IA plastic tube. The binding was measured as described under methods.

Table 1 shows a comparison of the consistency of binding of ^{125}I-hormone to a/s adsorbed to plastic tube directly or through -IA, -IAIA and -AIA bridges, along with the specific binding obtained with conventional method (double antibody-PEG). The consistency of binding is about ±4% for direct binding. In contrast the binding to tubes immunochemically coated with a/s was ±2%. This consistency is almost the same as that obtained for conventional liquid phase assay. Thus it is clear that the binding of ^{125}I-hormone to immobilised a/s on plastic through immunochemical bridge is highly consistent. It is also seen from the table that this consistency is not only seen for one hormone, but is true for hCG, oFSH and human luteinizing hormone (hLH).

Table 2 shows the stability of the a/s coated tubes (thorough -IA and -IAIA

Table 1. Comparison of ^{125}I-hormone binding to immobilised a/s through immunochemical bridges*.

Type of bridge	n	a/s adsorbed	^{125}I-hormone bound cpm ± SD	± SD (%)
Direct adsorption	12	hCG	19444 ± 1750	9·00
	30	oFSH	27268 ± 1055	3·87
Conventional binding	20	oFSH	22222 ± 459	2·07
	20	hCG	41483 ± 834	2·01
-IA bridge	18	hCG	29572 ± 335	1·13
	10	hLH	26387 ± 473	1·81
-AIA bridge	10	hCG	11537 ± 337	2·92
-IAIA bridge	10	hCG	14303 ± 283	1·98
	5	oFSH	30136 ± 212	0·70
	6	hLH	22645 ± 403	1·78

*Binding of hLH was done for hCG a/s adsorbed on the plastic tube. For hCG and oFSH, plastic tubes coated with corresponding a/s was used.

Table 2. Stability of a/s coated tube.

Days of storage	^{125}I-hormone* added (cpm)	^{125}I-hormone bound (cpm ± SD)	Per cent bound
hCG a/s coated tubes using -IA bridge			
3	54,000	14303 ± 283	26·5
16	48,000	14252 ± 295	29·6
34	52,000	12080 ± 284	23·2
45	60,000	17576 ± 305	29·3
oFSH a/s coated tubes using -IAIA bridge			
3	—	—	—
35	56,000	30136 ± 212	53·8
65	40,000	19798 ± 394	49·5
80	52,000	24520 ± 506	46·0

*Immunologically active.

cent bound is not significant considering that the ^{125}I-hormones used are different for each experiment. On storage the consistency of binding (SD) do not change, indicating that the a/s adsorbed on the plastic through immunochemical bridge is infact stable for atleast 90–100 days.

Comparison of the assay using immobilised a/s and normal a/s is shown in figure 3. With regard to sensitivity, binding and consistency the assays are comparable. In the inset of the figure 3 is shown the Scatchard plot of the two assays. The K_a and capacity are infact measurable. The apparent K_a in the solid phase appears to be rather low, compared to that in the liquid phase.

Competitive binding of ^{125}I-oFSH to immunochemically immobilised a/s on plastic tube (curve b) and normal a/s (curve a) is shown in figure 4. The profiles are similar and the duplicates are comparable to that of the conventional double antibody assay. Table 3 shows the consistency and parallelism obtained for the measurement of a control sample of oFSH in 5 determinations using the a/s coated

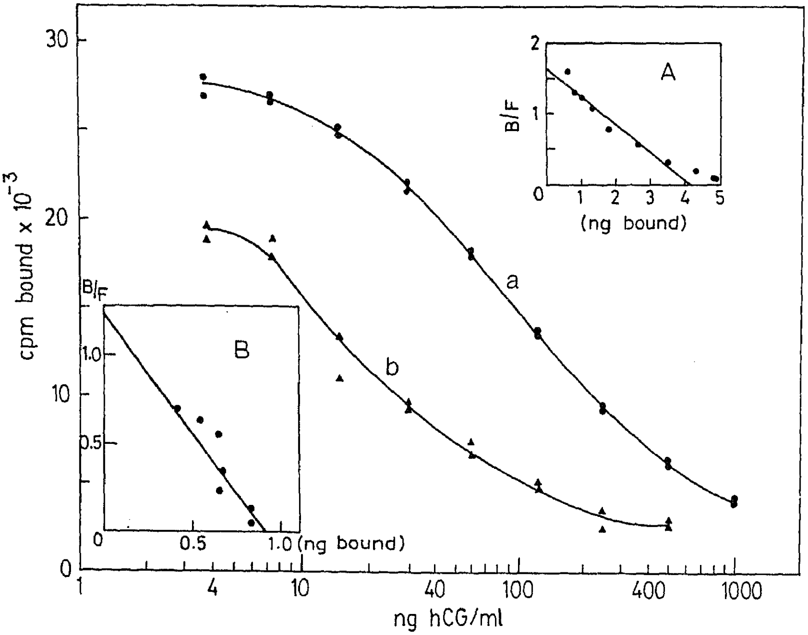

Figure 3. Displacement profile in RIA using immobilised antibody on plastic tube (curve a) and conventional method (curve b) for hCG. Tubes were coated with 1/200,000 a/s (0·6 ml) on -IA bridge. Liquid phase assay was done using 100 μl of 1/100,000 a/s in a total volume of 0·6 ml. ^{125}I-hCG added was 60,000 cpm in both cases. Insets A and B show the Scatchard plots for curves (a) and (b) respectively.

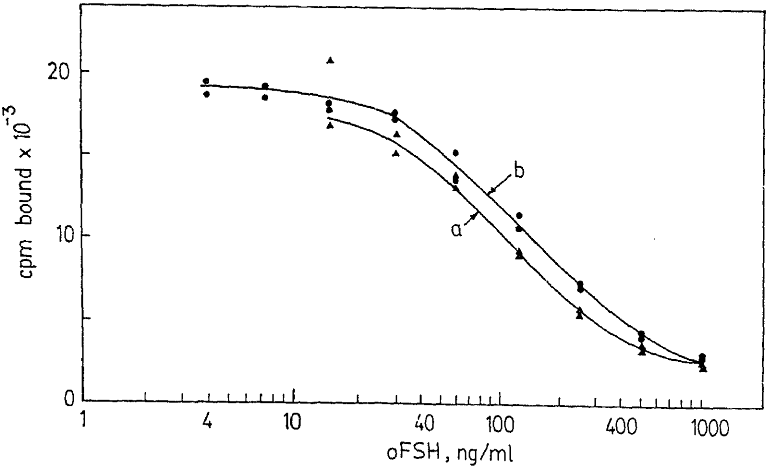

Figure 4. Displacement data of RIA using conventional method (curve a) and noncentrifugation method using immobilised a/s on plastic tube (curve b) for oFSH. Plastic tubes were coated with 1/100,000 a/s (0·6 ml) on -IAIA bridge. Liquid phase assay was done using 1/25,000 (100 μl) a/s. ^{125}I-oFSH added was 50,000 cpm per tube (-IAIA bridge).

Table 3. Analysis of tube RIA using immunochemically coated a/s on plastic tubes through -IAIA bridge.

Volume of control sample used	oFSH ng/ml read in assay nos 1	2	3	4	5	Mean ± SD
25 μl	44	55	50	50	55	51 ± 4
50 μl	100	100	120	120	110	110 ± 10
100 μl	240	230	250	230	250	240 ± 10
Mean	207	217	230	223	230	
± SD	30	15	26	21	17	

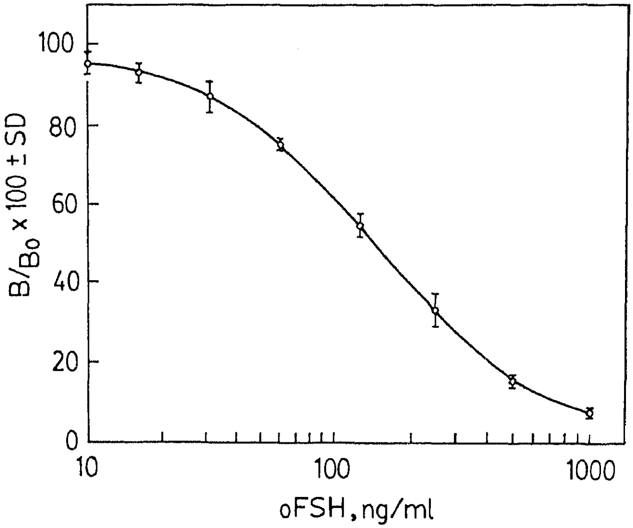

Figure 5. Displacement profile in oFSH RIA's using a/s (for oFSH) immobilised on plastic tube through -IAIA bridge. The -IAIA bridges were coated with 1/100,000 a/s (0·6 ml). A total of 5 assays were done over a period of 2 weeks using plastic tubes coated with the a/s. The bars show the SD of specific binding at each concentration of the hormone.

s. They are superimposable indicating good reproducibility of the assay. e 6 presents displacement analysis in hLH assay system using -IA coated a/s. in the figure shows the B/Bo *vs* concentration plot of 4 such assays (curve a). results clearly indicate that the solid phase assay is quite consistent and ducible and is independent of the nature of the bridge (-IA or -IAIA) used for dsorption of the a/s. Displacement of ^{125}I-hLH by a human serum is also 1 in the inset (curve b), which is parallel to normal displacement.

oles 4 and 5 show the effect of normal serum (human, sheep and rabbit) and ery of the exogeneously added hCG from serum samples. The recovery is itative with negligible interference from serum. Figure 7 (curves a, b and c) the displacement profile with immobilised a/s (on plastic through nochemical bridge) in the absence of serum (curve a), and presence of 100 μl of

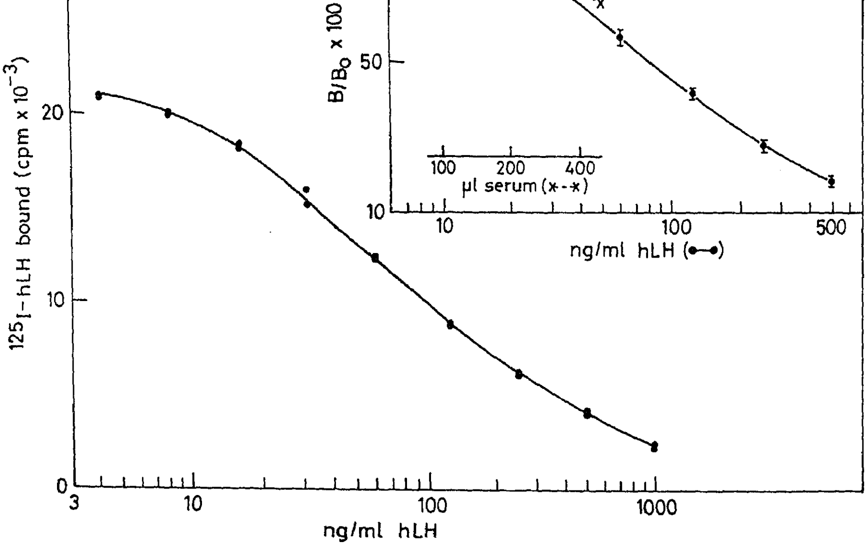

Figure 6. Representative displacement profile in solid phase RIA for hLH using immobilised a/s on -IA bridge. Inset shows the B/Bo *vs* concentration plot for 4 such assays (curve a). Displacement of ^{125}I-hLH in one of the assay by different volumes of a human serum sample obtained from WHO for a qc program (curve b). Vertical bars represents the ± SD at each concentration of the hormone used for displacement.

Table 4. Recovery of hormone (hCG) by RIA in the presence of normal sheep and rabbit serum (type of bridge used is -IA).

Serum added	hCG added exogeneously (ng)	Specific binding (%)	ng hormone recovered	Recovery (%)
Sheep (100 μl)	0	100*	—	—
	8	70·2	9·0	112
	31	44·7	34·0	109
	125	22·1	125·0	100
Rabbit (100 μl)	0	100*	—	—
	8	76·0	7·5	94
	31	50·2	27·0	87
	125	21·5	130·0	104

*Specific binding in the presence of sheep and rabbit serum with respect to the buffer, sp. binding was 94 and 97% respectively.

demonstrates that the binding characteristics of the ^{125}I-hCG to the adsorbed a/s is same in the absence and presence of serum samples. Table 6 shows the recovery of the exogeneously added hLH to human serum samples described under figure 7. Recovery is quantitative from serum samples carrying basal hLH also. Curve d in figure 7 and curve b in figure 6 show the displacement pattern by varying volumes of human serum samples and is parallel to normal displacement profile.

Table 5. Recovery of hormone (hCG) from human serum samples*.

No. of samples	Exogeneously added hCG (ng)	Recovery ng ± SD	Recovery (%)
6	200	210 ± 38	105
10	100	110 ± 20	105
2	50	60	120

*Random serum samples from different individuals. Specific binding of ^{125}I-hCG in the presence of these human serum samples was found to be 99·5 ± 5% (100 μl serum, n = 18) compared to the binding in the buffer (type of the bridge -IAIA).

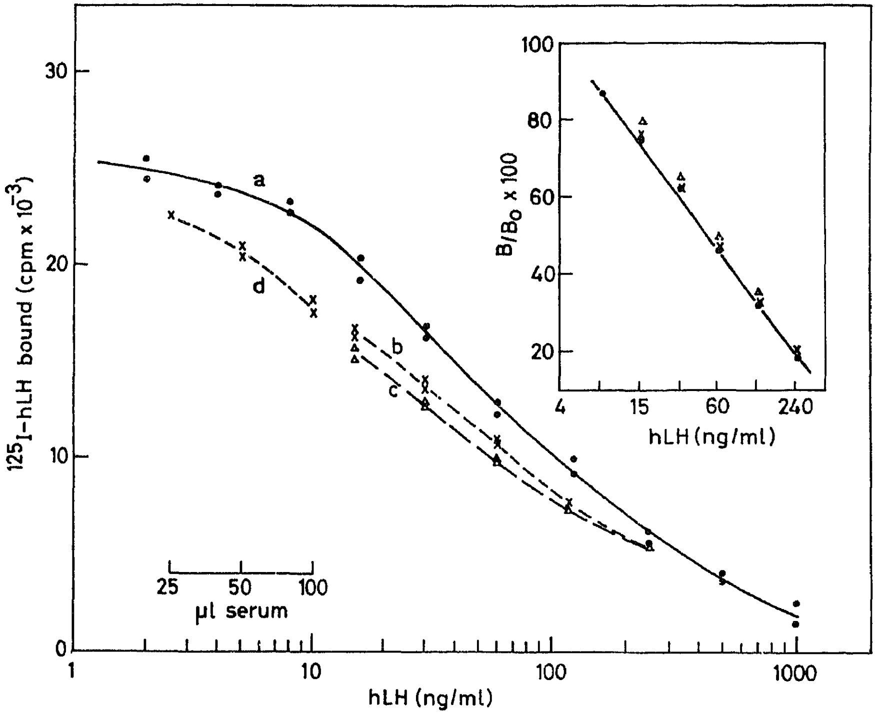

Figure 7. Displacement profile and recovery of hLH in solid phase RIA. The type of the bridge used was -IA. Curve (a), Normal displacement profiles; curves (b) and (c), displacement profiles in hLH RIA in the presence of 100 μl of two serum samples 1 and 2 with basal hLH levels of 11 and 19 ng/ml respectively; curve (d), displacement of ^{125}I-hLH by a human serum sample. Inset represents B/Bo*100 *vs* hLH concentration for curves (a), (b) and (c) respectively. The nature of the samples used is explained under table 6.

Table 7 shows a comparison of quantitative aspects of a/s adsorbed on the plastic tube either by direct coating or through -IA bridge. The results clearly demonstrate that the adsorption of a/s in both the direct method and through the bridge per tube corresponds to about 150–200 μl (1/100,000 dilution) and this gives about

Table 6. Recovery of hormone (hLH) from human serum samples containing basal hLH*.

Serum number	Exogeneously added hLH (ng)	Serum hLH (blank)	Total hLH measured (ng)	Recovery (%)
1	0	11	11	—
	15	11	30	113
	30	11	46	110
	60	11	70	97
	120	11	130	99
	250	11	250	96
2	0	19	19	—
	15	19	36	105
	30	19	55	112
	60	19	85	107
	120	19	150	108
	250	19	280	104

*Both the serum samples used were from two female volunteers during 10-14 days of the cycle. hCG a/s was used for the measurement of hLH using ^{125}I-hLH as tracer and hLH for displacement (type of the bridge -IA).

Table 7. Efficiency of a/s adsorption and comparison of ^{125}I-hCG binding between adsorbed and normal a/s.

Type of bridge	Vol. of a/s used for adsorption (μl-dilution)	a/s adsorbed[a]# (%)	Vol. of a/s adsorbed[b] (μl/dilution)	I-hCG bound cpm
Direct	600 – 1/20,000	5	150 – 1/100,000	28,000
-IA[c]	600 – 1/200,000	60	180 – 1/100,000	30,000
normal[d]	—	—	100 – 1/100,000	22,000

[a] This was calculated by the binding of ^{125}I-hCG to the a/s left after adsorption.

[b] This also represents the volume of the a/s that is used for binding the ^{125}I-hCG.

[c] Data for -IA and normal binding are taken from figure 3.

[d] This represents the binding that is obtained in the conventional binding done under same conditions.

the adsorption of a/s by immunochemical bridge is near quantitative (60%) compared to the direct method which is less than 5%.

Discussion

The data presented clearly demonstrates that the adsorption of antibody through immunochemical bridge is satisfactory. It is seen from table 1 that consistency of binding to immunochemically adsorbed a/s is comparable to the binding observed in the liquid phase (conventional method). The consistency is far superior to that observed with the tubes coated with a/s directly. This observation is true for several hormones as shown in the table (hCG, oFSH and hLH).

Immunochemically coated tubes are stable at 4°C for atleast 3 months, and probably longer. This observation makes it viable to have a large number of tubes coated and stored at 4°C and use it as required. Making -IA or -IAIA bridge is a batch operation and as many as 1000–2000 tubes can be coated within 4 h with minimal fatigue. In the whole operation the only place where care needs to be exercised is in the first step (addition of normal rabbit serum). Subsequent steps are much like in enzyme-linked immunosorbent assay operations.

Competitive binding analysis of direct a/s, and immunochemically adsorbed a/s on plastic tubes (table 2, figures 1 and 3) clearly show that duplicates in the conventional method and immobilised a/s method are comparable. The sensitivity of the assay using immunochemically coated tubes is of the same order as that of the conventional method.

Consistency of the tube assay is definitely acceptable (figures 5 and 6) where consolidated displacement analysis of 5 assays are presented. The mean values obtained within an assay and between assays is within limits as in the conventional method (222+10 ng/ml, and 217+15 ng/ml), Scatchard plot of displacement analysis by homogeneous (conventional) and plastic tube methods show that the plots are linear in both cases. However K_a in the plastic tube method appears to be little on the lower side. Apparent capacity for the plastic tube is about 4 ng/tube. Considering that the reaction between the hormone and antibody in the plastic tube assay is nonhomogeneous, neither the capacity nor the affinity can be taken seriously.

Interference from serum on specific binding to the coated a/s is insignificant. Recovery of the hormones added exogeneously to serum sample (human, sheep and rabbit, 100 μl) are quantitative clearly demonstrating that this method has a potential application in measuring hormones in serum samples. The fact that parallel displacement is seen for human serum sample in hLH measurement justifies the above conclusion (figure 7). In addition the binding characteristic of the ^{125}I-hLH to a/s coated tube is not affected by the presence of serum is clear from the inset in figure 3. The above conclusion is also drawn from the data shown in table 4 for sheep and rabbit serum. Thus serum interference in the assay is ruled out.

The above assay system is economical in terms of double antibody as well as primary a/s. The quantity of the double antibody required in this system per tube is 100-fold less than the requirement in the liquid phase assay. It is also very economical in terms of the a/s required. For example in hCG a/s the requirement in the liquid phase would be 100 μl of 1/100,000 dilution. The volume of a/s adsorbed immunochemically on the plastic tube is hardly equivalent to 150–200 μl (1/100,000; dilution-60% of 600 μl of 1/200,000 a/s is adsorbed), this giving about 20% more binding than in the liquid phase. In the direct method of coating 600 μl of 1/20,000 dilution of a/s is required which is about 30 times more, only about 5% or less being adsorbed, the remaining 95% becoming nonfunctional because of inactivation of a/s at such high dilutions in the absence of proteins. Such inactivation of a/s for both hCG as well as oFSH has been observed in our laboratory. Thus immunochemical method of adsorption of the a/s is extremely economical in terms of primary a/s in comparison to any other method of immobilisation known so far.

The type of immunochemical bridge does not appear to have any preference in RIA. The recovery of the hormone in -IA, -AIA and -IAIA bridges are all excellent, stability and consistency comparable and interference from serum sample insignificant. Hence technically there is little to choose among the 3. -IA bridge would require least manipulations and hence probably the most useful practically.

-IA bridge to rabbit and monkey systems have worked well (for hCG and oFSH respectively) and there is no reason to believe that other systems may present problems. Manipulation wise this method is also very simple. Once the coated tubes are available the operations are minimal involving addition of buffer, standard or sample and ^{125}I-hormone. There is no requirement of the centrifugation nor the manipulations for the separation of the bound hormone from the free hormone.

Thus a/s coated plastic tubes through immunochemical bridge has all the technical advantages of double antibody method. In addition it has the following other advantages: (i) requires 100-fold less of double antibody, (ii) does not require centrifugation, (iii) has much less experimental manipulation and (iv) extremely economical on the a/s, almost nearly the same as in the conventional liquid phase assay. Added to these practical advantages are that the a/s coated tubes are stable for atleast 90 days. Hence a mass scale preparation of a/s coated tubes can be undertaken.

On the basis of these data we believe that this method of RIA is an alternative to expensive double antibody method. The method retains all the technical excellence of the conventional method and possess other practical advantages of economy and ease of operation.

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Biochemical studies with bi-resistant mutants (ethambutol plus streptomycin and isoniazid plus streptomycin) of *Mycobacterium smegmatis* ATCC 607

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Abstract. Biochemical characteristics of bi-resistant mutants (resistant to ethambutol plus streptomycin or isoniazid plus streptomycin) of mycobacteria isolated by replica plating from *Mycobacterium smegmatis* ATCC were compared with those of the drug-susceptible strains. Reduced incorporation of [^{14}C]uracil, [^{3}H]lysine and [^{14}C]acetate into RNA, protein and phospholipids respectively was seen in the resistant mutants. Total phospholipids were enhanced in ethambutol plus streptomycin resistant mutant and decreased in isoniazid plus streptomycin resistant mutant. There were similar changes in levels of individual phospholipids. The resistant mutants revealed an accumulation of phospholipids in the cell wall, and a marked decrease of phospholipids in the cell membrane in comparison to the susceptible strain. Several qualitative alterations in the polypeptide profile (with respect to number and molecular weight) of the crude protein extract and of different subcellular compartments were seen in the resistant mutants.

Keywords. *Mycobacterium smegmatis*; isoniazid; ethambutol; streptomycin; cell membrane; cell wall; phospholipids.

Introduction

Tuberculosis chemotherapy regimens involve the concomitant administration of two or more antitubercular drugs because the emergence of organisms resistant to two or more drugs at the same time is less likely. Double-drug combination therapy is more effective than treatment with any single drug (Mitchison, 1984; Grosset *et al.*, 1985). The combination of isoniazid with streptomycin has been demonstrated to be almost as potent as the triple-drug combination of isoniazid–streptomycin–ethambutol (Tsang *et al.*, 1978). The mode of action of each of these drugs is known (Santhanam and Subramanian, 1977). The primary action of isoniazid is its inhibitory effect on mycolic biosynthesis in sensitive cells (Winder and Collins, 1970). Streptomycin affects protein synthesis at ribosomal level and inhibits chain elongation (Shaila *et al.*, 1975). Ethambutol interferes with nucleic acid metabolism (Tsukamura and Mizuno, 1972) as well as synthesis of mycolic acid (Kilburn and Takayama, 1981). Inspite of double-drug or triple-drug regimens, increased drug resistance of mycobacteria has been reported (Mitchison, 1984). Although sufficient information is available on the biochemical aspects of different antitubercular drugs, reports regarding the mechanism of drug resistance in mycobacteria, specially

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Abbreviations used: (INH + SM)R, Isoniazid plus streptomycin resistant; (EM + SM)R, ethambutol plus streptomycin resistant; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; CL, cardiolipin; PIMs, phosphatidylinositolmannosides; PE, phosphatidylethanolamine; TPL, total phospholipids double-drug-resistant mutants are limited. This communication deals with the biochemical variations in drug-resistant and susceptible strains of *Mycobacterium smegmatis* ATCC 607.

Materials and methods

Culture and growth of the organism

A culture of *M. smegmatis* ATCC 607 was maintained on Lowenstein–Jensen medium. The two bi-resistant mutants, one resistant to a combination of isoniazid (1000 μg/ml) and streptomycin (150 μg/ml), designated (INH + SM)R, and one resistant to a combination of ethambutol and streptomycin (100 μg/ml each), designated (EMB + SM)R, were isolated in the laboratory by replica plating from the wild strain (*M. smegmatis* ATCC 607) as described by Lederberg and Lederberg (1952). The stability of mutants resistant to drugs was confirmed by maintaining them on drug-free media for 2–3 months and then transferring them to medium containing drug(s). The mutants were maintained on solid Youman's medium in screw-capped vials with or without drug(s). The cultures were grown in the modified Youman's medium as stationary cultures at 37°C and harvested in the mid-log phase.

Macromolecular synthesis

The incorporation of [^{3}H]lysine (sp. activity 5600 mCi/mmol), [^{14}C]acetate (sp. activity 58·46 mCi/mmol) and [^{14}C]uracil (sp. activity 56 mCi/mmol) (Bhabha Atomic Research Centre, Bombay) into proteins, phospholipids and RNA was monitored. Mid-log-phase cells were harvested, washed with physiological saline, and resuspended in sterile Youman's medium. The cells were preincubated under shaking conditions at 37°C for 30 min. Radiolabelled precursor was then added and incubation was continued for 2 h at 37°C. The reaction was terminated and processed as described earlier (Khuller *et al.*, 1984).

Extraction and identification of lipids

Lipids were extracted and purified by the method of Folch *et al.* (1957). The individual phospholipids were separated by thin-layer chromatography (TLC) on silica gel H plates using the solvent system choloroform : methanol : 7 N ammonia (65 : 25 : 4, v/v/v). Phospholipids were quantitated by estimating lipid phosphorus according to the method of Bartlett (1959) as modified by Marinetti (1962).

Isolation of cell wall and cell membrane fractions

Cells were disintegrated by ultrasonication according to the method of Hill and Ballou (1966). Subcellular fractions were isolated and purified by differential centrifugation according to the method of Kearney and Goldman (1970). Purity of the subcellular fractions was assessed by electron microscopy and by measuring the activity of ATPase, a marker enzyme for cell membranes, according to the method of Penumarti and Khuller (1983).

Metabolism of phospholipids

Synthesis and degradation of phospholipids in mid-log-phase cells were examined by the pulse-chase technique of Kanfer and Kennedy (1963) using [^{32}P]orthophosphoric acid as described earlier (Mahajan and Khuller, 1984).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done on slab gels by the method of Laemmli (1970). The gels were scanned densitometerically on an LKB 2202 Ultroscan laser densitometer at 750 nm at a scanning speed of 80 mm/min.

Results and discussion

Development of resistance to the combination of drugs retarded the growth (figure 1) of both the mutants, *viz.*, (INH+SM)R and (EMB+SM)R. The log phase was prolonged, as has been reported for *Psuedomonas aeruginosa* resistant to quarternary ammonium compound (Anderes *et al.*, 1971). Therefore susceptible cultures

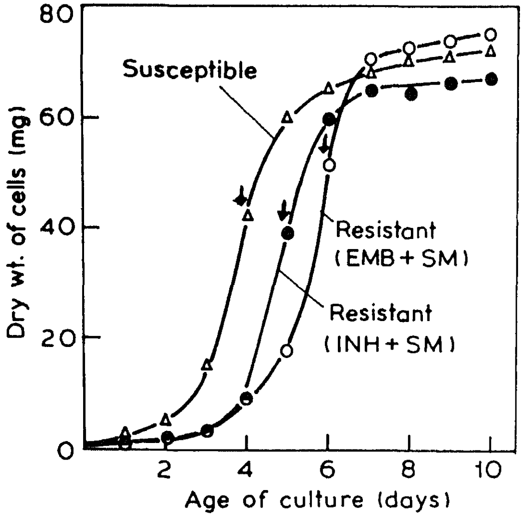

Figure 1. Growth patterns of susceptible and resistant cultures of *M. smegmatis*. Arrows indicate time of harvesting of cells.

monitored in order to examine macromolecular synthesis in susceptible and resistant cultures (table 1). In comparison with the susceptible strain, the (INH + SM)R mutant showed an approximately 8-fold (~88%) decline in [^{14}C]uracil incorporation, and ~50% decrease in labelled lysine and acetate incorporation. Similarly, the (EMB + SM)R mutant revealed a 70% decrease in RNA synthesis, while protein and phospholipid synthesis were decreased by 50%. The reduced incorporation of all 3 radiolabelled precursors indicates lower metabolic status of the mutants.

Table 1. Incorporation of [^{14}C]uracil, [^{3}H]lysine and [^{14}C]acetate into macromolecules of drug-susceptible and resistant mutants of *M. smegmatis*.

Cell type	[^{14}C]uracil (cpm/mg protein)	[^{3}H]lysine (cpm/mg protein)	[^{14}C]acetate (cpm/mg of lipid phosphorus)
Susceptible	42,950 ± 6,400	2,270 ± 120	30,410 ± 3,950
(EMB + SM)R	12,780 ± 580***	970 ± 100***	15,820 ± 580***
(INH + SM)R	5,320 ± 410***	1,110 ± 120***	14,520 ± 380***

Values are mean ± SD of 3 independent batches analysed in triplicate.
***$P < 0{\cdot}001$.

The total phospholipid content of the bi-resistant mutant (INH + SM)R was significantly decreased in comparison with that of the susceptible strain (table 2). The decrease cannot be attributed to resistance to isoniazid or streptomycin alone as monoresistant mutants of *M. smegmatis* to INH and SM did not reveal any change in total phospholipids (Kanwar and Khuller, 1988). In contrast, in the (EMB + SM)R mutant a marked increase in the total phospholipid content was apparent. Increased lipid content has been suggested to be responsible for drug resistance in other microorganisms (Mackenzie and Jordan, 1970; Norrington and James, 1970; Chang *et al.*, 1972). Individual phospholipids analysed were significantly decreased in (INH + SM)R and increased in (EMB + SM)R, in agreement with decrease/increase in total phospholipid content. The decrease was most prominent in cardiolipin (CL), followed by phosphatidylinositolmannosides (PIMs) and phosphatidylethanolamine (PE) (table 2). Alterations in levels of individual phospholipids may be due to altered activities of the particular phospholipid metabolizing enzymes.

Table 2. Phospholipid composition of drug-susceptible and resistant mutants of *M. smegmatis*.

		Phospholipid composition		
Cell type	Total phospholipid (mg/g dry wt of cells)	PIMs	PE (mg/g dry wt of cells)	CL
Susceptible	25·45 ± 0·40	11·51 ± 0·93	3·07 ± 0·69	10·92 ± 0·06
(EMB + SM)R	31·02 ± 0·66	15·33 ± 0·98**	5·92 ± 0·78**	9·14 ± 0·32
(INH + SM)R	14·52 ± 0·70***	7·11 ± 1·63**	1·83 ± 0·21*	5·13 ± 1·13***

Values are mean ± SD of 3 independent batches analysed in triplicate.
*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Since changes were observed in phospholipid composition, metabolism of phospholipids was examined. Net synthesis of phospholipids was reduced in both mutants (figure 2). The distribution of radiolabel in individual phospholipids after a 120 min pulse indicated that in the mutants PE synthesis was highest, followed by PIMs and CL; in the susceptible cells sequence (decreasing order of net synthesis) was CL, PIMs and PE, in agreement with the observations of Dhariwal *et al.* (1978). The increase in the net synthesis of PE with concomitant decrease in the net synthesis of cardiolipin in the mutants suggest that these phospholipids are possibly synthesized by a common pathway, as hypothesized for phospholipid synthesis in *E. coli* (Miyazaki *et al.*, 1985; Kobayashi *et al.*, 1986).

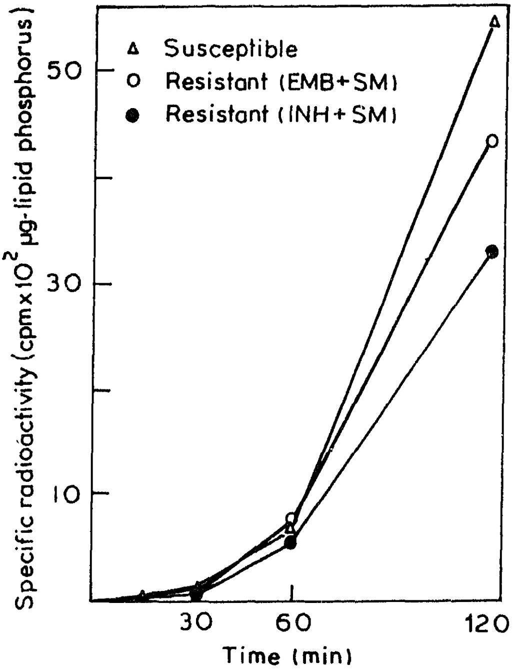

Figure 2. Specific radioactivity incorporated into phospholipids of susceptible and resistant cultures of *M. smegmatis*.

Following the pulse of radioactive precursor, the label was chased for 24 h after removing extracellular [^{32}P]orthophosphate. In susceptible cells, increased incorporation was observed up to 2 h, followed by loss of radioactivity. In resistant cells, there was continuous incorporation up to 4–8 h, followed by loss during the subsequent period, probably because of slow utilization of labelled precursors in these cells. Increased degradation of phospholipids was seen in the (INH + SM) R mutant, compared to the susceptible strain, while the (EMB + SM)R mutant showed decreased degradation (data not presented). These results suggest that the alterations seen in total phospholipid content (table 2) are due to changes in degradation rate rather than synthesis.

The subcellular distribution of phospholipids was investigated. It is evident from table 3 that both cell wall and cell membrane of *M. smegmatis* contain phospho-

Table 3. Phospholipid composition (mg/g dry weight of cell fraction) of subcellular fractions of drug-susceptible and resistant mutants of *M. smegmatis.*

	Cell type		
	Susceptible	(EMB + SM)R	(INH + SM)R
Cell wall			
TPL	25·35 ± 1·79	63·83 ± 7·57***	39·45 ± 1·34***
PIMs	14·95 ± 1·64	43·46 ± 4·06***	25·87 ± 2·51***
PE	1·76 ± 0·31	9·40 ± 1·25***	3·46 ± 0·94*
CL	8·09 ± 1·12	10·65 ± 1·85*	11·47 ± 1·37**
Cell membrane			
TPL	60·17 ± 0·46	39·45 ± 1·34***	41·62 ± 1·57***
PIMs	37·18 ± 1·30	17·97 ± 0·53***	29·38 ± 2·40**
PE	8·30 ± 0·68	3·62 ± 0·42***	4·82 ± 10·39***
CL	14·70 ± 0·79	8·78 ± 1·22***	6·58 ± 1·00***

Values are mean ± SD of 3 different batches analysed in triplicate.
*$P<0·05$; **$P<0·01$; ***$P<0·001$.

lipids and that the latter has comparatively more phospholipid. This distribut
phospholipids in the wall and membrane fractions of the susceptible strain
agreement with the earlier results of Kates (1964), Asselineau (1966) and Ma
and Khuller (1983). As no report is available on phospholipid compositi
subcellular fractions in drug-resistant mutants of mycobacteria, the present
have been compared with those for other microorganisms. Both the double-re
mutants showed significant alterations in total and individual phospholipid c
of the cell wall, like Gram-negative antibiotic-resistant bacteria (Mackenzi
Jordan, 1970). These findings in general support the hypothesis that the amo
phospholipids in the cell wall plays a role in determining the spectrum o
sensitivity by an exclusion mechanism (Brown, 1971). In contrast to the situat
the cell wall, total phospholipid content of the cell membrane fraction was
derably decreased in the mutants, as in polymyxin-resistant *Pseudomonas aeru*
(Brown and Wood, 1972) and penicillin-resistant gonococci (Mavrov *et al.*, 1

In keeping with the alterations in the total phospholipid (TPL) content
wall and cell membrane in the mutants, the levels of PIMs, PE and CL wer
elevated in the cell wall and decreased in the cell membrane. Elevation of P
in the cell wall is of significance as polar lipids have a prominent role
formation of a cell surface structure that regulates permeability (Alberghina,
Alterations in PE content may possibly modulate surface charge in drug-re
mutants.

The protein profiles of the drug-susceptible and resistant strains were analy
SDS-PAGE. Qualitative changes were seen in the polypeptide profiles of
protein extract, cell wall and cell membrane of the mutants, implying that
components are involved in the drug resistance. These observations are in accor
with those made on *Acromonas salmonicida* (Wood *et al.*, 1986).

The observations presented here clearly demonstrate that development o
resistance in mycobacteria leads to alterations in the composition of cell wa

Acknowledgement

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Impairment of alternate pathway (CD2) of T cell activation in leprosy

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Abstract. Recent studies in basic immunology have been directed towards the understanding of the mechanism of T cell activation. T cells can be activated to proliferate *via* the classical pathway through the antigen receptor (CD3-Ti) or *via* the alternate pathway through the CD2 receptor. Since immunologic unresponsiveness in lepromatous leprosy is considered to be due to the inability of T cells to proliferate upon stimulation, we have been interested in the nature of these receptors and the activation pathways in lymphocytes of leprosy patients. In the present investigation we demonstrate: (i) CD2 receptor (E-receptor) is downregulated in bacterial index positive lepromatous leprosy patients. (ii) The alternate pathway of T cell activation is impaired in lepromatous patients as revealed by the inability of their lymphocytes to proliferate in response to a pair of mitogenic anti-CD2 monoclonals. (iii) The addition of recombinant interleukin 2 *in vitro* restores the ability of lymphocytes from lepromatous patients to proliferate in response to anti-CD2 antibodies. (iv) Interestingly, CD2 modulation and the associated functional impairment could be brought about in peripheral blood lymphocytes from normal subjects by prior treatment with *Mycobacterium leprae in vitro*. This approach would be useful in understanding the molecular events leading to the defective T cell functions in leprosy.

Keywords. Leprosy; CD2; CD3; IL2; *Mycobacterium leprae.*

Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. One population of leprosy patients, referred to as lepromatous type (LL), is known to have reduced T cell functions as evidenced by *in vitro* and *in vivo* tests (Nath *et al.*, 1977; Godal, 1978). Though several approaches (Bloom and Mehra, 1984; Kaplan and Cohn, 1985; Nath *et al.*, 1984) have been used in attempts to understand the mechanism of immunologic unresponsiveness in lepromatous leprosy (LL) patients, none has yet gained wide acceptance.

Earlier studies indicated that the number of T cells, as enumerated by sheep erythrocyte-rosette forming cell (E-RFC) assay (Dwyer *et al.*, 1973; Nath *et al.*, 1977; Jaswaney *et al.*, 1980), was reduced in LL patients. Subsequently, when monoclonal antibody to a pan-T-cell marker (CD3) was used with the immunofluorescence technique, the proportion of T cells was found to be normal (Bach *et al.*, 1981; Mshana *et al.*, 1982). We have recently repeated these experiments and found that, while the proportion of CD3-positive cells was normal, E-receptor (CD2) positive cells were significantly reduced in LL patients (Muthukkaruppan *et al.*, 1987). On the basis of our findings in conjunction with the role of CD2 receptor in T cell biology (Springer *et al.*, 1987), we have proposed a hypothesis to explain the mechanism of immunologic unresponsiveness in leprosy (Muthukkaruppan, 1986).

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Abbreviations used: LL, Lepromatous leprosy; E-RFC, erythrocyte-rosette forming cell; LLBI+, bacillary index positive LL; IL2, interleukin 2; LLBI−, bacillary index negative LL; PBL, peripheral blood lymphocytes; rIL2, recombinant IL2; LFA3, lymphocyte function associated antigen 3.

Our earlier studies (Muthukkaruppan *et al.*, 1987) have clearly demonstrated that (i) the expression of CD2 receptor, but not that of CD3, was downregulated in bacillary index positive LL (LLBI+) patients, (ii) this downregulation was strongly associated with reduced proliferative response to mitogens and antigens, and (iii) CD2 modulation and the associated immunosuppression could be brought about even in lymphocytes from normal subjects by prior incubation with *M. leprae* (Dharmendra lepromin) *in vitro*.

CD2 receptor is expressed on all T cells (Kamoun *et al.*, 1981). Certain monoclonal antibodies to $T11_1$ epitope of CD2 receptor were shown to downregulate T cell proliferation by blocking the production of interleukin 2(IL2) (Palacios and Martinez-Maza, 1982; Tadmori *et al.*, 1985; Gromo *et al.*, 1987). On the other hand, pairwise combinations of anti-CD2 monoclonal antibodies to certain other epitopes ($T11_2$ and $T11_3$, or $T11_1$ and D66) can induce T cells to proliferate (Meuer *et al.*, 1984; Brottier *et al.*, 1985). CD2 receptor also helps in thymocyte–thymic epithelial interaction (Singer and Haynes, 1987). These findings suggest that CD2 can function in activation signalling (alternate pathway of T cell activation) as well as in cell-to-cell adhesion.

The present study was directed at understanding the nature of the alternate pathway of T cell activation in leprosy. Our data indicate that the alternate pathway of T cell activation is impaired in lymphocytes of LL patients as a result of CD2 modulation by *M. leprae* and this impairment in proliferation is due to a block in IL2 secretion.

Materials and methods

Patients

A total of 19 lepromatous and 8 tuberculoid leprosy patients who were attending Arogya Agam, Leprosy Hospital, Andipatty, were studied. These patients were graded by Ridley–Jopling classification (Ridley and Jopling, 1966) on the basis of bacillary smears and clinical symptoms. The LL patients were subdivided into LLBI+ *i.e.* those who demonstrated acid-fast bacilli in slit-skin smears, and bacillary negative (LLBI−) patients. Most of the patients were undergoing multi-drug therapy. Control subjects were healthy volunteers from the university campus.

Mycobacterial preparations

Dharmendra lepromin was kindly provided by Dr U. Sengupta, Central JALMA Institute for Leprosy, Agra. Bacille Calmette-Guerin (BCG) was obtained from BCG Vaccine Laboratories, Madras, and subjected to treatment similar to the preparation of Dharmendra lepromin (Sengupta *et al.*, 1978).

Indirect immunofluorescence assay

The percentages of CD2+ and CD3+ cells were enumerated as already described

(Muthukkaruppan *et al.*, 1987). To 1×10^6 peripheral blood lymphocytes (PBL) in 100 μl of medium monoclonal antibodies OKT3 (for CD3) and OKT11 (for CD2) (Orthodiagnostic Inc., USA) were added and the cell suspensions incubated at 4°C for 30 min with frequent mixing. After washing the cells in RPMI 1640 (Sigma), 50 μl of goat anti-mouse IgG FITC conjugate (Coulter Electronics) were added and the tubes were incubated at 4°C for 30 min. The percentage of fluorescence-positive cells was determined by phase contrast/fluorescence microscopy, counting 200 cells per tube. Ascites fluid (BRL) was used as control for monoclonal antibodies.

To study the effect of *M. leprae* on the modulation of CD2, 1×10^6 PBL from healthy non-contacts were incubated at 37°C for 24 h with Dharmendra lepromin containing 3×10^5 bacilli. As a control, PBL from the same individuals were treated with the same number of BCG separately. After this treatment cells were washed and stained for the detection of CD3 and CD2 receptors.

Lymphocyte transformation test

Triplicate cultures of PBL from leprosy patients were prepared in flat-bottom 96-well culture plates (Greiner, FRG) and cells were stimulated with optimal concentration of phytohaemoagglutinin-M (Bacto, USA) or monoclonal antibodies $T11_1$ and D66 (a kind gift from Dr Alain Bernard, France) either alone or together as previously described (Brottier *et al.*, 1985). In another set of cultures, PBL from healthy non-contacts were pretreated with *M. leprae* or BCG (1×10^5 bacilli per well at 37°C for 24 h) before adding mitogen or antibodies.

Addition of recombinant IL2

Recombinant IL2 (rIL2), a kind gift of Dr Sinigaglia, Hoffman La Roche, Basel, Switzerland, was added at 50 units per ml to cultures, wherever mentioned.

Statistics

Statistical comparisons were made by Student's *t* test.

Results

Alternate pathway of T cell activation in leprosy

We have used a pair of mitogenic anti-CD2 monoclonal antibodies ($T11_1$ and D66) to elucidate the proliferative response of lymphocytes *via* the alternate pathway of T cell activation (figure 1). PBL from healthy non-contacts exhibited a good proliferative response. However, only a minimal response (SI 7·7 ± 2·8) was observed in LLBI+ patients, in contrast to the response in borderline tuberculoid and LLBI− patients. Comparable results were obtained when PHA-M was used as a stimulant. Indirect immunofluorescence assay with PBL from the above subjects revealed a significant reduction in the proportion of CD2-positive cells only in LLBI+ patients (figure 1). These results indicate that the alternate pathway of T cell activation is impaired in LLBI+ patients and that this is correlated with the

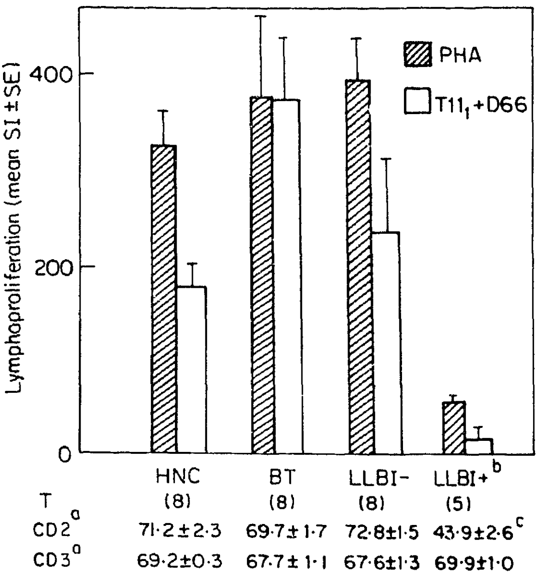

Figure 1. Impairment of alternate pathway of T cell proliferation in leprosy in correlation with CD2 modulation. Figures in parentheses below the histograms are numbers of individuals studied. HNC, Healthy non-contacts; BT, borderline tuberculoid. [a]Percentages of immunofluorescence-positive cells ± SE; [b]$P<0{\cdot}001$ between stimulation indices (SI) of LLBI+ (BI ranging from 0·4–6) and LLBI− patients; [c]$P<0{\cdot}001$ between CD2-positive and CD3-positive cells.

Effect of M. leprae on alternate pathway of T cell activation in PBL of healthy non-contacts

The reduction in the proportion of CD2-positive cells was found in LLBI+ patients, *i.e.* in persons harbouring *M. leprae in vivo*. We therefore wanted to determine the effect of *M. leprae* on PBL of healthy non-contacts. For this purpose we have developed an *in vitro* test in which PBL from healthy non-contacts were treated with *M. leprae* or BCG and the expression of CD2 and CD3 receptors was analysed. As shown in table 1, *M. leprae*, but not BCG, significantly modulated the

Table 1. Percentage of CD2-positive and CD3-positive cells in normal PBL treated with *M. leprae* or BCG.

	Immunofluorescence-positive cells[b] (%)	
Treatment[a]	CD2	CD3
No treatment	71·44 ± 1·80	69·34 ± 1·50
M. leprae	38·18 ± 4·53[c]	67·28 ± 1·94
BCG	70·16 ± 3·28	70·70 ± 3·05

[a]Number of subjects 5 in each group.

[b]Cells were stained with monoclonal antibodies OKT11 for CD2 and OKT3 for CD3. The data are mean ± SE.

[c]$P<0{\cdot}001$ between untreated and *M. leprae* treated

expression of the CD2 receptor, while the percentage of CD3-positive cells remained unaltered. In parallel experiments, PBL from the healthy subjects were incubated with *M. leprae* or BCG for 24 h and then stimulated with a pair of anti-CD2 antibodies (figure 2). The data clearly indicate impairment of the alternate pathway of T cell activation as a result of CD2 modulation. The response to PHA-M was also suppressed by *M. leprae*, as shown in figure 3.

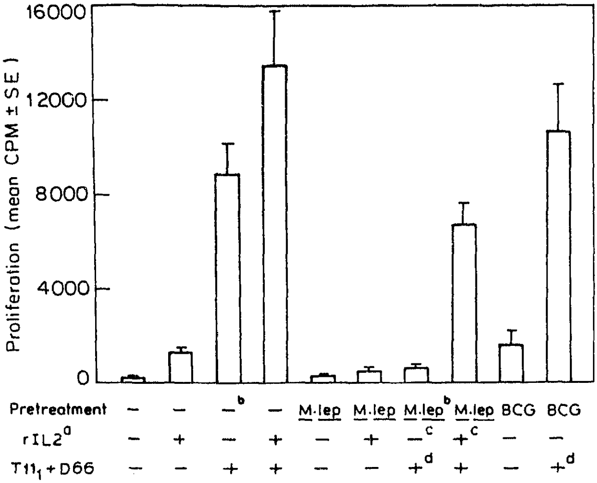

Figure 2. Impairment of alternate pathway of T cell proliferation in PBL of healthy non-contacts by *M. leprae in vitro*. [a]IL2 and/or $T11_1+D66$ were added to untreated PBL cultures or cultures pretreated for 24 h with *M. leprae*/BCG; [b]$P<0\cdot001$ between cpm of *M. leprae* treated and untreated cultures stimulated with $T11_1+D66$; [c]$P<0\cdot001$ between cpm of *M. leprae* treated cultures stimulated with $T11_1+D66$ with and without the addition of rIL2; [d]$P<0\cdot001$ between cpm of *M. leprae* and BCG treated cultures stimulated with $T11_1+D66$.

Recovery of proliferative response by the addition of rIL2

We next examined the mechanism of unresponsiveness produced by *M. leprae* through CD2 modulation. Table 2 shows that the addition of rIL2 (50 U/ml) could restore the proliferative response to the pair of anti-CD2 antibodies in PBL of LLBI+ patients. Further, *M. leprae* pretreated PBL from healthy non-contacts also showed recovery of the proliferative response to anti-CD2 antibodies upon the addition of rIL2 (figure 2). Interestingly, very little proliferative response to *M. leprae* antigens was observed in cultures of normal PBL even with the addition of rIL2 (figure 2).

Discussion

Several studies have clearly demonstrated that the cell-mediated immune response is suppressed in LL patients (Godal, 1978; Nath, 1983; Bloom and Mehra, 1984).

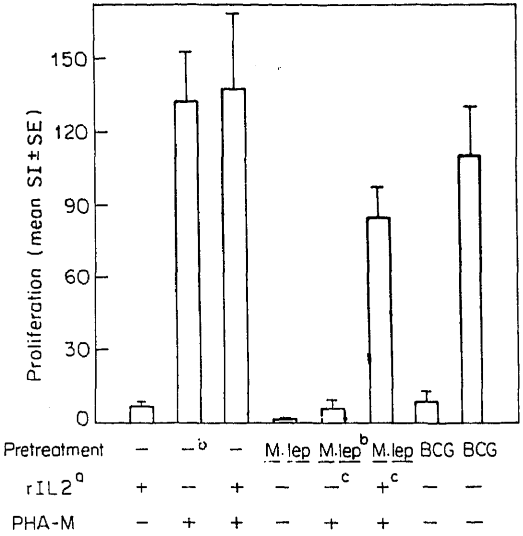

Figure 3. Impairment of proliferative response to PHA-M in PBL of healthy non-contacts by *M. leprae in vitro.* [a]IL2 and/or PHA-M were added to PBL untreated cultures or cultures pretreated for 24 h with *M. leprae*/BCG; [b]$P<0{\cdot}001$ between SI of *M. leprae* treated and untreated cultures stimulated with PHA-M; [c]$P<0{\cdot}001$ between SI of *M. leprae* treated cultures stimulated with PHA-M with and without the addition of rIL2.

Table 2. Recovery of proliferative response *via* alternate pathway by the addition of rIL2 to LLBI+ PBL cultures.

Subject[a] (LLBI+)	IL2	Mean incorporation of radio-labelled thymidine (cpm) Medium	D66+T11$_1$	PHA
1.	–	164	1988	7044
	+	3202	28206	115168
2.	–	303	2865	12853
	+	937	33650	75508
3.	–	479	863	3549
	+	421	22683	14987
4.	–	457	6182	10559
	+	580	37159	24829

[a]At the time of study, the bacillary index of the patients ranged between 0·4 and 5. The percentage of CD2-positive and CD3-positive cells were 46·0 ± 1·8 and 62·2 ± 2·6 respectively ($P<0{\cdot}001$).

to *M. leprae*. This was suggested to be due to reduced IL2 production and IL2 receptor expression (Nath *et al.*, 1984; Kaplan and Cohn, 1985; Mohagheghpour *et al.*, 1985). We have initiated a new approach to explain the mechanism of *M. leprae* specific unresponsiveness in leprosy (Muthukkaruppan, 1986)

The CD2 receptor was found to be significantly downregulated in LLBI+ patients and this condition was strongly associated with impaired proliferative response to PHA-M and PPD (Muthukkaruppan *et al.*, 1987). The modulation of CD2 and the associated impairment of T cell proliferation can be brought about even in lymphocytes from normal subjects by prior incubation with *M. leprae* (Dharmendra lepromin) *in vitro* (Muthukkaruppan *et al.*, 1987). It is of interest to note that tuberculoid leprosy, though caused by the same bacteria, did not show reduction in the proportion of CD2-positive cells in the peripheral blood circulation (Nath *et al.*, 1977; Muthukkaruppan *et al.*, 1987). This means that the CD2 modulation would require the presence of a certain detectable level of bacilli, as in LLBI+ patients.

Our more recent study (Muthukkaruppan *et al.*, 1988) indicates that the CD3 receptor, though apparently expressed in normal proportion in the peripheral blood of LLBI+ patients, is functionally impaired, since CD2 modulated T cells did not proliferate in response to mitogenic anti-CD3 monoclonal antibody. The data presented here demonstrate the functional impairment of the alternate pathway (CD2) of T cell proliferation, since PBL from LLBI+ patients were unable to proliferate in response to a pairwise combination of anti-CD2 monoclonal antibodies (figure 1). Thus, both classical (CD3) and alternate (CD2) pathways of T cell activation are impaired as a result of CD2 modulation. Further, proliferative response to *M. leprae* could not be recovered even by the addition of IL2. However, when such T cells were activated by a potent stimulus like PHA-M or a pair of anti-CD2 monoclonals vigorous proliferation ensued only when exogenous IL2 was present in the cultures (figures 2 and 3).

Accumulating evidence in recent years suggests the importance of a functional interrelationship between CD2 and CD3 receptor complexes in T cell activation (Breitmeyer, 1987). T cells after treatment with certain anti-CD2 monoclonals were unable to proliferate in response to mitogenic anti-CD3 antibodies. Furthermore, the importance of interaction between CD2 receptor and its natural ligand, lymphocyte function associated antigen 3 (LFA3), in T cell functions has been emphasized (Krensky *et al.*, 1983; Springer *et al.*, 1987). In the light of these findings, the present study may suggest the importance of CD2 modulation in unresponsiveness in leprosy. However, further investigations are required to understand the mechanism of CD2 modulation by *M. leprae* as well as the nature of CD2 and IL2 receptor expression in LLBI+ patients.

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Correlation of bacterial viability with uptake of [^{14}C]acetate into phenolic glycolipid-1 of *Mycobacterium leprae* within Schwannoma cells

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Abstract. The viability of *Mycobacterium leprae*, maintained within 33B Schwannoma cells, was estimated in terms of incorporation of [^{14}C]acetate into its specific phenolic glycolipid-1. This measure of viability was correlated with two other assays, *viz.*, fluorescein diacetate/ethidium bromide staining and mouse footpad growth. Observation of a 2-fold increase in the number of intracellular *Mycobacterium leprae* over an experimental period of 12 days also corroborated this contention. Furthermore, on addition of anti-leprosy drugs to these intracellular *Mycobacterium leprae* there was significant decrease in phenolic glycolipid-1 synthesis indicative of loss of viability of the organisms. This study also established the importance of the host cell for active bacillary metabolism, as *Mycobacterium leprae* maintained in cell-free conditions showed no incorporation into phenolic glycolipid-1. Moreover, compromising the host's protein synthesis capacity with cycloheximide, also led to reduction in bacillary metabolism. As this system measures the metabolic synthesis of a unique *Mycobacterium leprae* component, it would be useful for development and screening of compounds acting against specific bacillary targets.

Keywords. *Mycobacterium leprae*; Schwannoma cell line; lipid metabolism.

Introduction

Mycobacterium leprae, an intracellular parasite, displays a unique affinity for the Schwann cells of the peripheral nerves (Iyer, 1965; Job, 1979) which they infect and in which they multiply. *In vitro* studies have been successful in demonstrating the limited multiplication of *M. leprae* within murine Schwann cells (Mukherjee and Antia, 1985). Within such host cells, *M. leprae* synthesize a specific component, phenolic glycolipid-1 (PGL-1), a dimycocerosyl phthiocerol with a unique trisaccharide component (Hunter and Brennan, 1981). This has been demonstrated in macrophages (Ramasesh *et al.*, 1987) and in a glial cell line (Mukherjee *et al.*, 1985). A distinct advantage of monitoring PGL-1 synthesis is that it is unique to the bacillus and can be readily distinguished from contaminating host components. However, it was imperative to first determine whether the synthesis of PGL-1 as measured by incorporation of radioactive acetate into the molecule reflects viability

In the present study, therefore, incorporation of [^{14}C]acetate into PGL-1 of intracellular *M. leprae* has been assessed as a measure of bacterial viability. Schwannoma cells were selected as hosts in preference to normal Schwann cells, for the availability of bulk material. Synthesis of PGL-1 was also studied in bacilli whose viability was compromised by addition of both bacteriostatic and cidal drugs, *viz.*, 4,4′-diaminodiphenyl sulphone (DDS) and rifampicin (RFP). The viability of *M. leprae* as estimated by incorporation of ^{14}C-acetate into PGL-1 was correlated with other tests established for bacterial viability *viz.*, fluorescein diacetate/ethidium bromide (FDA/EB) staining and mouse footpad (MFP) growth. Further, the role played by the host cells in the maintenance of activity of these intracellular pathogens was examined by inhibiting their protein metabolism using cycloheximide. Results indicated that incorporation of ^{14}C-acetate into PGL-1 correlated well with viability of the organism; this phenomenon being depressed in nonviable, drug-treated bacilli.

Materials and methods

Chemicals.

The culture medium, Dulbecco's minimum essential medium (DMEM) and foetal calf serum (FCS) were purchased from GIBCO Laboratories, USA, while nutrient agar and Lowenstein-Jensen media were obtained from Hi-media, Bombay. Silicic acid was from Loba-Chemie Indoaustranal Co., Bombay, and silica gel from Acme Synthetic Chemicals, Bombay. Florisil (100–200 mesh), RFP and cycloheximide were purchased from Sigma Chemical Co, St. Louis, Missouri, USA. DDS was obtained from Burroughs Wellcome Co., Bombay. All the solvents used were of Analar grade obtained from Glaxo Laboratories, Bombay.

Host cells

The 33B glial cell line originally derived from an ethylnitrosurea-induced tumour in Wistar-Furth rats (Fields *et al.*, 1975) maintained in our laboratory was used as the host cell. These cells were maintained in a growth medium consisting of DMEM containing 10% FCS and antibiotics. When these cells formed a confluent sheet, the cultures were irradiated at 4000 rads to stop further multiplication.

Inoculation of M. leprae

A suspension of *M. leprae* was prepared from infected armadillo tissue by homogenization and sequential centrifugation to minimize tissue debris (Ambrose *et al.*, 1978). Bacillary count was determined using the method described by Hart and Rees (1960). The presence of other mycobacterial contaminants was checked by inoculation onto nutrient agar and Lowenstein-Jensen media.

Irradiated Schwannoma cultures were inoculated with freshly isolated *M. leprae* (5×10^6/ml) and incubated at 37°C for 72 h which was found optimal for phagocytosis. The unphagocytosed *M. leprae* were then removed by repeated washing. One set was inoculated with bacilli which were killed by formalin-fixation for 24 h.

Another control used was freshly harvested *M. leprae* maintained in the above mentioned culture medium consisting of FCS-enriched DMEM in the absence of host cells. These bacilli were incubated with 0·5 μCi/ml [^{14}C] acetate for the same duration of 12 days at 37°C.

Radioactive counts from cultures not infected with *M. leprae* were subtracted from those of experimental cultures, to minimize host contamination.

Acetate incorporation

After removal of non-phagocytosed *M. leprae*, [1, 2-C-14] acetate (0·5 μCi/ml) (LCC-161, sodium acetate, sp. act. 60·3 mCi/mmol, Bhabha Atomic Research Centre, Bombay) was added to all cultures. The culture medium containing radiolabelled acetate was replaced twice a week and cultures were maintained for a total period of 12 days.

Addition of drugs

Prior to the addition of radiolabelled precursor to the *M. leprae* infected 33B cell cultures, anti-leprosy drugs, *viz.*, DDS (5 μg/ml) and RFP (5 μg/ml) were added to their respective cultures which were then incubated at 37°C for 12 days in the presence of [^{14}C]acetate.

Effect of cycloheximide

Cultures infected with *M. leprae* were treated with 3 μg/ml cycloheximide along with [^{14}C]acetate. Cultures not infected with *M. leprae* served as controls. All cultures were fed twice a week, each time with fresh drug and label and maintained for 12 days.

Incorporation into PGL-1 was compared between untreated and drug or cycloheximide-treated cultures.

Termination and processing of cultures

For termination of the cultures, the cells were detached from the culture surface using 0·25% trypsin. The bacilli were released by sonication at 23 KHz in a Soniprep 150 ultrasonic disintegrator for 2 min. The host cell debris was removed by centrifugation at 220 g for 10 min. The supernatant obtained was centrifuged at 10,000 *g* for 45 min to obtain the *M. leprae* in an enriched form, relatively free from tissue contamination. This bacillary pellet was washed twice with phosphate-buffered saline before estimating the bacillary count. Control cultures were processed identically.

Lipid extraction and estimation

chloroform to remove out the neutral lipids and 2 and 5% methanol in chloroform, to obtain the glycolipids. These latter fractions contained the PGL-1 component. This eluant was dried and resolved by thin-layer chromatography (TLC) on silica gel G coated plates (thickness 0·5 mm) using chlorofom:methanol:water (90:10:1, v/v/v) as the solvent system. Incorporation into PGL-1 was estimated from the spot with an R_f value corresponding to standard PGL-1 on a Kontron 300 Scintillation counter. Counts were expressed as disintegration per minute (dpm) for 10^6 bacilli.

Purification and characterization of PGL-1

PGL-1 was extracted in larger quantities by preparative TLC. Its presence was confirmed using α-naphthol spray reagent following TLC. Authenticity of this component was determined by (i) indirect immunofluorescence staining using the monoclonal antibody raised against the specific trisaccharide moiety (antibody 46·7 from Dr. Bloom, Albert Einstein, USA), (ii) enzyme-linked immunosorbent assay (ELISA) using the above mentioned antibody and (iii) comparison of IR absorbtion spectra, carried out on a Perkin-Elmer 684 spectrophotometer using methylene chloride as the solvent (Hunter and Brennan, 1981).

Visual quantitation

33B cultures on coverslips were infected with $0{\cdot}5 \times 10^6$ *M. leprae*/ml for 24 h to permit minimal phagocytosis. Cultures were terminated at various time intervals up to 12 days. These cultures were stained by the Ziehl-Neelsen method and the number of bacilli per cell estimated.

M. leprae viability assays

FDA/EB staining: The protocol followed was similar to that adapted by Ramasesh *et al.* (1984) from the original method of Kvach *et al.* (1984), for intracellular bacilli. The viable bacilli which are able to convert the FDA to fluorescent fluorescein by the action of active esterases appear green when viewed under UV light. Staining was carried out on *M. leprae* within 33B cell cultures terminated at various time intervals. Per cent viability was calculated by comparison of 33B cells containing green fluorescing bacilli (GFB) to those containing acid fast bacilli (AFB) from identical cultures stained by the Ziehl-Neelsen method.

M. leprae growth in MFP: The procedure described by Rees (1964) was followed. *M. leprae* released from 33B cells after 12 days in culture were inoculated into MFP to check for growth. Freshly harvested armadillo *M. leprae*, inoculated directly into MFPs served as control.

Statistical analysis: For tabular representations, dpm values or the percentages are expressed as mean ±SE. The paired Student's *t* test has been employed for assessment of significance values.

Results

Characterization of PGL-1

PGL-1 extracted from 33B cell-resident *M. leprae* was characterized by comparison of its IR spectrum with that of standard PGL-1. An ELISA using the extracted PGL-1 as antigen and the monoclonal antibody raised against the specific trisaccharide, established its identity. Further, immunofluorescence staining of intracellular *M. leprae* using the above-mentioned monoclonal antibody also confirmed the presence of PGL-1.

Incorporation of [^{14}C]acetate into PGL-1 fraction of intracellular M. leprae

Increased incorporation of [^{14}C]acetate into the PGL-1 fraction of intracellular *M. leprae* was noted over a period of 15 days (table 1). This demonstrated the active synthesis of PGL-1 in this system.

Table 1. Relationship between duration of [^{14}C]acetate labelling and its incorporation into PGL-1 of *M. leprae.*

Days of incubation	Radioactivity in PGL-1 (dpm)
5	3,648 ± 256
10	6,913 ± 717
15	10,919 ± 669

Data are the mean of 3 sets of cultures expressed for 10^6 *M. leprae.*

Determination of viability of M. leprae within 33B cells

The experiments mentioned below were designed to correlate uptake of [^{14}C]acetate with bacterial viability.

The number of *M. leprae* within 33B cell cultures was visually counted after Ziehl-Neelsen staining over a period of time. As observed from table 2, the number

Table 2. Determination of viability of intracellular *M. leprae* by visual quantitation following Ziehl–Neelsen staining.

Days post-infection	AFB/cell
0	8·8 ± 1·1
6	11·4 ± 2·0
12	19·1 ± 0·5

Results are expressed as the mean ± SE of duplicates from 3 sets of of AFB/cell increased 2-fold after 12 days of incubation. This indicated that intracellular *M. leprae* are maintained in a structurally integral form during the course of the experiment. The fact that these organisms were viable was obtained from the following two assays.

(i) Cultures of *M. leprae* within 33B cells were also stained using the FDA/EB assay to determine their viability. Results showed that per cent viability of these bacilli increased up to 12 days in culture, (table 3a) confirming that the 33B cells were suitable for the maintenance of viable and metabolically active *M. leprae*.

Table 3a. Viability of intracellular *M. leprae* ascertained over a period of 12 days using the FDA/EB technique.

Days post infection	Viability (%)
0	64·1 ± 3·9
6	66·4 ± 2·4
12	83·3 ± 2·5

$$\%\ \text{viability} = \frac{\text{no. of 33B cells containing GFB}}{\text{no. of 33B cells containing AFB}} \times 100.$$

(ii) Bacilli inoculated into MFPs, after incubation within 33B cells showed the characteristic growth curve of *M. leprae* (table 3b), confirming the viability of these intracellular organisms.

Table 3b. The MFP harvest counts obtained for *M. leprae* released from 33B cells after 12 days of incubation.

Duration of food-pad inoculation (months)	Control	Bacillary count Set I	 Set II
0	$1{\cdot}0 \times 10^4$	$1{\cdot}0 \times 10^4$	$1{\cdot}0 \times 10^4$
6	$1{\cdot}3 \times 10^5$	$2{\cdot}4 \times 10^5$	$1{\cdot}2 \times 10^5$
7	$1{\cdot}5 \times 10^5$	—	$1{\cdot}1 \times 10^5$
8	$4{\cdot}5 \times 10^4$	$3{\cdot}0 \times 10^4$	—
10	—	—	$1{\cdot}8 \times 10^5$
12	$2{\cdot}7 \times 10^5$	$1{\cdot}8 \times 10^5$	—

Each value is the mean from 4 smears.
(—), No bacilli observed.
Control, *M. leprae* isolated from armadillo.
Sets I and II, *M. leprae* derived from 33B cells.

Effect of drugs on PGL-1 synthesis

Earlier studies using both MFP and FDA/EB have demonstrated that drug concentrations equivalent to 5 μg/ml or even less, are effective in decreasing the viability of intracellular *M. leprae* (Holmes and Hilson, 1972; Bhagria and Mahadevan, 1987). Incubation of *M. leprae* infected 33B cell cultures in the decrease in the incorporation of [^{14}C]acetate into PGL-1, implying its lowered synthesis and a decrease in viability (table 4).

Table 4. [^{14}C]Acetate incorporation into PGL-1 of *M. leprae.*

ML		FF		DDS		RIF	
X	%	X	%	X	%	X	%
4,128	100	586	14·20	—	—	283	6·83
235	100	131	55·75	—	—	—	—
618	100	138	22·33	—	—	183	29·61
2,550	100	—	—	1,089	42·71	980	38·43
51,602	100	23,765	46·05	33,993	65·88	2,635	5·12
458	100	—	—	212	46·29	—	—
458	100	—	—	368	80·35	—	—
Mean %	100	34·58 ± 9·77		58·81 ± 6·68		20·01 ± 8·30	
t value		$P<0·01$		$P<0·02$		$P<0·005$	

(—), Not done.
X, dpm obtained for 10^6 *M. leprae* after subtraction of counts for control cultures.
%, Calculated as experimental value divided by control value (for *M. leprae*) multiplied by 100.
ML, *M. leprae* (control).
FF, Formalin-fixed *M. leprae.*
DDS, *M. leprae* in the presence of DDS.
RFP, *M. leprae* in the presence of RFP.

Control cultures utilising formalin-fixed *M. leprae* were simultaneously maintained to correlate incorporation of radiolabel with viability. Significantly reduced, dpm counts were obtained in the PGL-1 fraction (table 4).

Role of host cells

The incorporation of radiolabelled acetate into PGL-1 maintained within Schwannoma cells was compared with that of *M. leprae* maintained in growth medium in the absence of host cells. In the latter case, no radiolabel was detected even though a spot corresponding to PGL-1 was observed following TLC, indicating a requirement of the host cell for the recording of active bacterial lipid metabolism. These results were corroborated by compromising the protein synthesis capacity of the 33B cells, using cycloheximide. A significant decrease was obtained in PGL-1 synthesis of the cycloheximide treated cultures (table 5), indicating that a metabolically competent host cell is essential for the survival of these bacilli.

Discussion

The highlight of this present study is the observation that incorporation of [^{14}C]

Table 5. Role of host cells—incorporation of [^{14}C]-acetate into PGL-1 in the presence of cycloheximide.

ML		CY	
X	%	X	%
412	100	233	5·64
235	100	38	16·17
618	100	41	6·63
2550	100	114	0·05
Mean %	100		7·12 ± 2·23
t value			$P<0{\cdot}005$

X, dpm counts for 10^6 bacilli in experimental cultures after subtraction of counts for control cultures.

%, Per cent incorporation as compared with control cultures.

ML, Control, *M. leprae* infected cultures:

CY, *M. leprae* infected cultures in the presence of cycloheximide.

bacterial drugs. Of the two anti-leprosy drugs, the reduction in incorporation was noted to be greater with RFP than with DDS, probably because RFP is bactericidal while DDS being bacteriostatic may not affect lipid synthesis to the same magnitude. Hence, the sensitivity of this system as a drug-screening assay may be less for static drugs. As a control, *M. leprae* killed by formalin-fixation were added to 33B cells. In this set, significantly lowered incorporation was observed, however, detectable radioactivity was recovered from the PGL-1 spot. This residual activity could be attributed to non-specific acetylation of long chain portions of this lipid, as formalinization may not be sufficient to inactivate all the enzymes. Uptake into PGL-1 of formalin-killed *M. leprae* has also been reported by others (Ramasesh *et al.*, 1987). Earlier studies by us were carried out using heat-killed bacilli as control, however, these gave extremely variable counts due to non-specific adsorption of radiolabel, and hence were discontinued.

An important observation of this study was the inability to incorporate [^{14}C] acetate into PGL-1 by *M. leprae* maintained in cell-free conditions. Incorporation of [^{14}C]palmitic acid into this component has nevertheless, been reported for *M. leprae* in axenic medium (Franzblau *et al.*, 1987). This may be indicative that a host cell milieu is necessary for the initial synthesis of fatty acids from free acetate. The importance of the host cells was further supported by observations on addition of the protein synthesis inhibitor, cycloheximide to the Schwannoma cells. Results demonstrated that incapacitating the host cell protein synthesis significantly decreased *M. leprae* metabolism, as reflected in reduction in incorporation of [^{14}C]acetate into bacterial PGL-1.

The host cells used in the present study were the 33B Schwannoma cells. These cells had to be irradiated to prevent their rapid multiplication, in order to provide sufficient time for a slow-growing organism like *M. leprae* to adapt to the host environment. The easy availability of these cells was an advantage, as it eliminated the dependence on other less accessible tissues like human and mouse macrophages, as utilised in other radiolabel assays (Prasad and Nath, 1981; Mittal *et al.*, 1983;

permitted the maintenance of simultaneous, identical, uninfected controls, whose radioactive counts were subtracted from those of experimental infected cultures, thus minimizing even the residual contamination by the host cells.

Besides its specificity, monitoring of the synthesis of PGL-1 has some advantages in the application of this system for screening of compounds that could act on the novel intermediates in the PGL-1 synthesis pathway. More generally, this system also appears to be capable of evaluating drugs that do not interfere directly with bacterial lipid metabolism.

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haracterization of cytosolic phosphoglucoisomerase from immature heat (*Triticum aestivum* L.) endosperm

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Abstract. Phosphoglucoisomerase from cytosol of immature wheat endosperm was purified 650-fold by ammonium sulphate fractionation, isopropyl alcohol precipitation, DEAE-cellulose chromatography and gel filtration through Sepharose CL-6B. The enzyme, with a molecular weight of about 130,000, exhibited maximum activity at pH 8·1. It showed typical hyperbolic kinetics with both fructose 6-P and glucose 6-P with K_m of 0·18 mM and 0·44 mM respectively. On either side of the optimum pH, the enzyme had lower affinity for the substrates. Using glucose 6-P as the substrate, the equilibrium was reached at 27% fructose 6-P and 73% glucose 6-P with an equilibrium constant of 2·7. The $\Delta F'$ calculated from the apparent equilibrium constant was $+597$ cal mol^{-1}. The activation energy calculated from the Arrhenius plot was 5500 cal mol^{-1}. The enzyme was completely inhibited by ribose 5-P, ribulose 5-P and 6-phosphogluconate, with K_i values of 0·17, 0·25 and 0·14 mM respectively. The probable role of the enzyme in starch biosynthesis is discussed.

Keywords. Wheat; *Triticum aestivum*; endosperm; cytosol; phosphoglucoisomerase.

troduction

arch is synthesized from sucrose in the endosperm of developing grains (Singh id Mehta, 1986). The process of sucrose–starch conversion has been studied quite tensively in grains of several cereals including wheat, with respect to both changes the activities of the enzymes involved and the concentration of metabolites umar and Singh, 1980, 1984). It is believed that the synthesis of starch from crose is sequentially indirect, temporally separated and compartmentalized IacDonald and ap Rees, 1983; Singh and Mehta, 1986). Sucrose, on entering the idosperm, is converted to triose-P in the cytosol, while starch biosynthesis from iose-P occurs in amyloplasts, which are membrane-bound, metabolically active id probably semi-autonomous organelles like chloroplasts (Macherel *et al.*, 1985; owokinos *et al.*, 1985; Dowson Day *et al.*, 1988; Entwistle *et al.*, 1988; gernprasirtsiri *et al.*, 1988). Hence, in developing wheat endosperm, some of the izymes of glycolysis and/or gluconeogenesis are present both in cytosol and in nyloplasts (Anand and Singh, 1985; Sangwan and Singh, 1987a, b, 1988). In a cent report, we have shown the presence of two forms of phosphoglucoisomerase -glucose-6-phosphate ketoisomerase, EC 5·3·1·9) (PGI) in developing endosperm wheat (Sangwan and Singh, 1987a). Here we report the detailed kinetic characristics of the cytosolic form of this enzyme from developing wheat endosperm.

laterials and methods

hemicals

l the biochemicals used in the present investigation were purchased from Sigma

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breviations used: PGI, Phosphoglucoisomerase; TEA, Triethanolamine

Chemical Co., St Louis, Missouri, USA. All other chemicals used were of high purity analytical grade obtained from BDH and/or E. Merck.

Plant material

Immature wheat grains were harvested from field-grown wheat crop (cv. WH-157) at day 26 after anthesis. Endosperms were removed and stored in liquid nitrogen for further use.

Enzyme purification

Unless stated otherwise, all steps involved in enzyme extraction and purification were carried out at 0–4°C.

Enzyme extraction: Immature wheat endosperms (30 g) were homogenized using pestle and mortar in 150 ml of 40 mM Tris–ethanolamine–NaOH (TEA) buffer (pH 7·1). The homogenate was squeezed through 4 layers of cheesecloth and centrifuged at 15,000 *g* for 45 min. The supernatant obtained was employed for ammonium sulphate and isopropyl alcohol fractionations.

$(NH_4)_2SO_4$ and isopropyl alcohol fractionations: Solid ammonium sulphate was added to the crude extract to 40% saturation. After 1 h, the precipitate was removed by centrifugation at 15,000 *g* for 30 min and the supernatant brought to 60% saturation by adding more ammonium sulphate. The precipitate, again removed as above, was dissolved in the minimum amount of extraction buffer and dialysed against 30 mM TEA buffer (pH 7·1) for 48 h. The dialysed ammonium sulphate fraction was then subjected to chilled (−12°C) isopropyl alcohol precipitation between 40 and 65% saturation. The precipitate obtained was collected as above and redissolved in the extraction buffer.

DEAE-cellulose chromatography: The fraction obtained between 40 and 65% saturation of isopropyl alcohol was applied to a DEAE-cellulose column (2·2 × 30 cm) previously equilibrated with the extraction buffer. The column was eluted with about two bed volumes of the buffer, followed by stepwise NaCl gradient elution. Fractions (5 ml each) containing PGI activity were pooled and concentrated by ultra-filtration (Amicon membrane filter).

Sepharose CL-6B chromatography: The above fraction was further purified by passing it through a column of Sepharose CL-6B (2·2 × 70 cm) pre-equilibrated with the extraction buffer. The enzyme was eluted at a flow rate of 20 ml h^{-1} with the equilibration buffer. The active fractions (3 ml each) were pooled and stored at 4°C for further studies.

Assay of PGI

The enzyme was assayed as described earlier (Sangwan and Singh, 1987a). The assay mixture, in a final volume of 1·4 ml, contained: 50 mM Tris–HCl buffer (pH 8·1), 1 mM fructose 6-P, 0·24 mM NADP$^+$, 2 units of glucose 6-P dehydrogenase and 100 μl of appropriately diluted enzyme preparation. The reaction was started by adding the substrate.

With glucose 6-P, the enzyme was assayed colorimetrically (Takeda *et al.*, 1967). The enzyme activity has been expressed in nkatals. One nkat is defined as the amount of enzyme that transforms one nmol of substrate per second.

Protein was estimated by the method of Lowry *et al.* (1951). However, in chromatography fractions, it was estimated by measuring absorbance at 280 nm.

Results and discussion

The enzyme preparation obtained between 40 and 65% isopropyl alcohol, when chromatographed through a DEAE-cellulose column, was resolved into two peaks of activity. The peak I enzyme did not adsorb to the column, whereas the peak II enzyme adsorbed and was eluted with elution buffer containing 0·1 M NaCl. No further enzyme activity was eluted up to 0·5 M NaCl. On rechromatography, the two peaks eluted at their respective salt concentration. The relative proportions (9:1) of the two forms did not change after incubating the homogenate at room temperature for various periods of time, thus excluding the possibility that the enzyme heterogeneity was due to proteolysis. Based on physical and kinetic characteristics, peak I enzyme was identified as cytosolic and peak II enzyme as amyloplastic PGI (Sangwan and Singh, 1987a). The cytosolic form, when further passed through a column of Sepharose CL-6B, yielded specific activity that was 650-fold higher than that of the crude homogenate (table 1). These results have been described in detail in our earlier report (Sangwan and Singh, 1987a).

Table 1. Purification of cytosolic PGI from immature wheat endosperm.

Fraction	Total activity (nkats)	Total protein (mg)	Sp. activity (nkats/mg protein)	Fold purification	Yield (%)
Crude extract	8688	1689	5·1	—	100
Ammonium sulphate	7819	284·2	27·5	5·4	90·0
Isopropyl alcohol	6519	99·2	65·7	12·9	75·0
DEAE-cellulose	3848	1·99	1938·8	380	44·3
Sepharose CL-6B	3531	1·063	3321·1	651	40·6

The enzyme was stable for more than a month at 4°C. It did not lose any activity on incubation for 15 min at 40°C. However, at higher temperatures, the enzyme was not stable and lost 50% of the activity at 45°C. At 55°C, the enzyme was completely inactivated. The activation energy, calculated from the Arrhenius plot, was 5500 cal mol^{-1}.

The enzyme had a molecular weight of about 130,000, as determined by gel filtration chromatography. A similar value (120,000–130,000) has been reported for the enzyme from germinating and developing *Cassia* seeds (Lee and Matheson, 1984), castorbean endosperm (Dennis *et al.*, 1985) and spinach leaves (Schnarrenberger and Oeser, 1974).

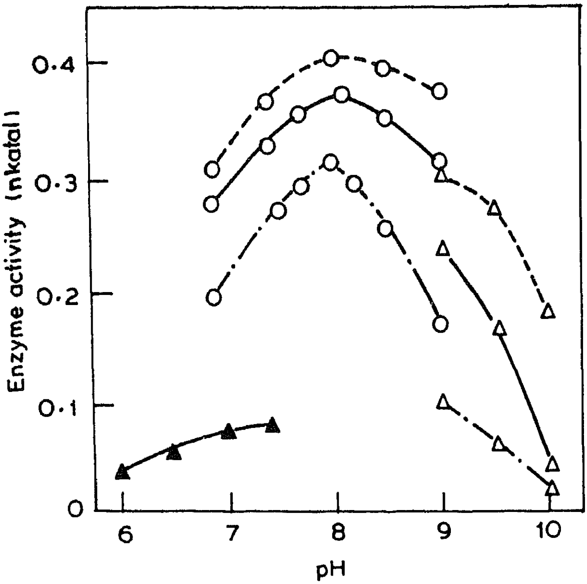

Figure 1. pH optimum curves of PGI at different substrate (fructose 6-P) concentrations: 0·4 mM (......), 1·0 mM (———) and 3·0 mM (– – –). Buffers: Tris–maleate (▲), Tris–HCl (○), glycine–NaOH (△).

in Tris–maleate buffer, as has been shown earlier for pea seed enzyme (Takeda *et al.*, 1967). The enzyme from developing *Cassia* seeds had good activity over a broad pH range (6–11). However, the spinach leaf enzyme had different pH optima with different substrates—7·6 with fructose 6-P and 8·6 with glucose 6-P (Schnarrenberger and Oeser, 1974).

At its optimal pH, the enzyme showed normal Michaelis–Menten kinetics with either fructose 6-P or glucose 6-P as the substrate. However, the enzyme had higher affinity for fructose 6-P than for glucose 6-P. The K_m values determined from Lineweaver–Burk plots were 0·18 and 0·44 mM respectively for fructose 6-P and glucose 6-P. These values are comparable with those reported for the enzyme from germinating *Cassia* seeds (Lee and Matheson, 1984) and sweet potato (Sasaki *et al.*, 1972). However, much higher values have also been reported for the enzyme from spinach leaves (Schnarrenberger and Oeser, 1974). There was a great influence of pH on K_m for fructose 6-P. Suboptimal pH, on either side of the optimum, increased K_m and decreased $V_{\max}$ (figure 2). The effect was more pronounced at alkaline pH than at acidic pH. The equilibrium constant calculated from K_m and $V_{\max}$ in both directions approached the ratio of 3:1, which is similar to the one reported for the enzyme from animal sources (Kahana *et al.*, 1960).

Figure 3 shows the equilibrium at 30°C using glucose 6-P as the substrate. The equilibrium was reached at 27% fructose 6-P and 73% glucose 6-P. The pea seed enzyme showed equilibrium at 35% fructose 6-P and 65% glucose 6-P (Takeda *et al.*, 1967). The apparent free energy change ($\Delta F'$) for formation of fructose 6-P, calculated from the apparent equilibrium constant ($K_{eq} = F6P/G6P$), was found to be $+597$ cal mol^{-1}. The value is rather high compared to that reported for pea seed

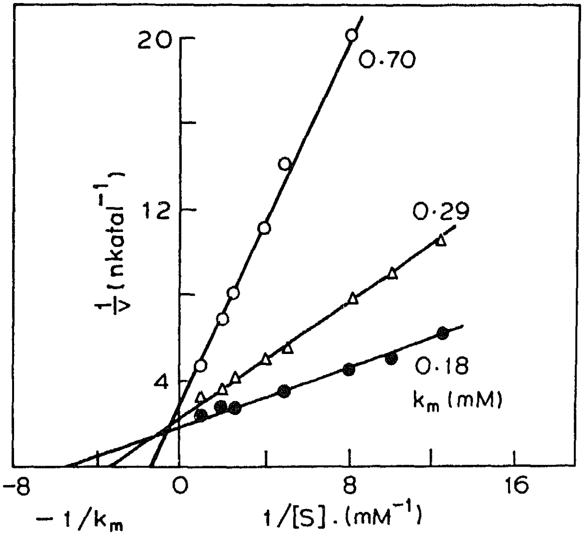

Figure 2. Effect of pH on K_m of PGI for fructose 6-P. Tris-HCl, pH 7 (△), Tris–HCl, pH 8·1 (●), and glycine–NaOH, pH 9·5 (○).

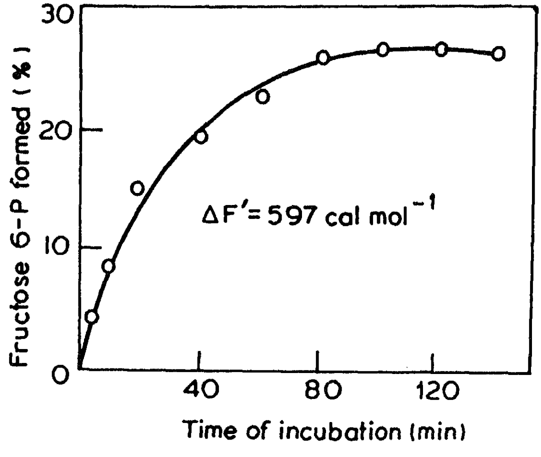

Figure 3. Equilibrium of interconversion of fructose 6-P and glucose 6-P by PGI at pH 8·1 and 30°C. The enzyme was incubated with 5 mM glucose 6-P.

The enzyme was inhibited competitively by 6-phosphogluconate (figure 4), ribose 5-P and ribulose 5-P, with K_i values, determined by Dixon's plot, of 0·14, 0·25 and 0·17 mM respectively. The enzymes from maize endosperm (Salamini *et al.*, 1972), *Cassia* (Lee and Matheson, 1984) and pea seeds (Takeda *et al.*, 1967) are also known to be inhibited by 6-phosphogluconate.

The organic acids succinate, acetate, citrate, tartarate, lactate, fumarate, glutamate, aspartate, glycollate, β-hydroxybutyrate, pyruvate and malate had no effect on the activity of the enzyme up to 10 mM. However, at this concentration, 2-oxoglutarate, oxaloacetate, malonate and maleate inhibited the activity by 20, 25,

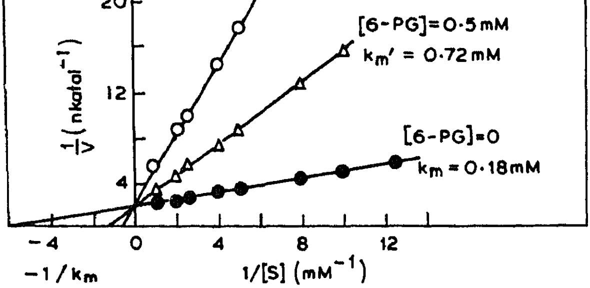

Figure 4. Competitive inhibition of purified PGI at various fixed concentrations of 6-phosphogluconate, with varying fructose 6-P concentrations.

phoglyceric acid at 1 mM concentration had no effect on the activity of the enzyme. Similarly, glucose 1,6-bisphosphate and fructose 2,6-bisphosphate at 50 μm concentration did not inhibit the enzyme.

The monovalent cations K^+, Na^+ and NH_4^+ (at 10 mM) did not influence the activity of the enzyme. The activity of the lucerne enzyme was also not affected by monovalent cations (McCleary and Matheson, 1976). Among the divalent cations, Mg^{2+}, Ca^{2+} and Ba^{2+} had no effect, whereas Cd^{2+}, Mn^{2+} and Zn^{2+} inhibited the enzyme to the extent of 15–20%, Sr^{2+} and Ni^{2+} inhibited the activity to the extent of about 45%. Hg^{2+} at 10 mM caused 80% inhibition. However, the lucerne enzyme was shown to be completely inactivated by 1 mM of Hg^{2+}. The maize enzyme has also been shown to be inhibited by Zn^{2+} (Salamini *et al.*, 1972).

The enzyme was insensitive to 10 mM concentration of Cl^-, Br^-, CO_3^- and SO_4^{2-}. However, inorganic phosphate and pyrophosphate could inhibit the enzyme to the extent of about 35%.

According to the currently held view, starch in the endosperm of developing grains is synthesized in the amyloplasts (Singh and Mehta, 1986). Sucrose, on entering the endosperm, is converted to fructose and UDP-glucose by sucrose synthase. Both these products are ultimately converted to fructose 6-P, the former by the action of fructokinase and the latter by the combined action of UDP-glucose pyrophosphorylase, phosphoglucomutase and phosphohexose isomerase. Fructose 6-P is then converted to triose-P by the well-known reactions of glycolysis in the cytosol. Triose-P then enters the amyloplasts *via* the phosphate translocator and is then ultimately converted to glucose 1-P by the reverse reactions of glycolysis. Glucose 1-P in plastids is converted to the precursor of starch synthesis (ADP-glucose) by the action of ADP-glucose pyrophosphorylase. All these enzymes have now been shown to be present in developing endosperm of cereal grains (Singh and Mehta, 1986) including wheat (Kumar and Singh, 1980, 1984) (figure 5).

According to the above scheme of reactions, we must expect at least two isoenzymic forms of triose phosphate isomerase, aldolase, fructose-1,6-bisphosphatase, phosphohexose isomerase and phosphoglucomutase. Such distribution of isoenzymic forms has now been well documented for the developing endosperm of wheat (Anand and Singh, 1985; Sangwan and Singh, 1987a, b; Sangwan and Singh,

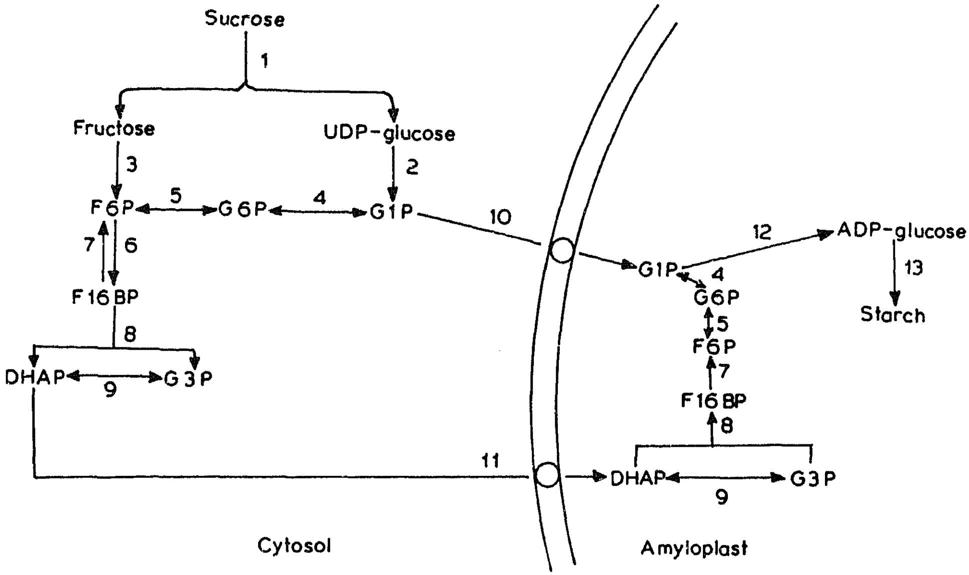

Figure 5. Proposed metabolic pathway of starch synthesis in amyloplasts of developing cereal grains. 1, Sucrose synthase; 2, UDP-glucose pyrophosphorylase; 3, hexokinase; 4, phosphoglucomutase; 5, phosphohexose isomerase; 6, ATP-dependent phosphofructokinase; 7, fructose-1,6-bisphosphatase; 8, aldolase; 9, triose-phosphate isomerase; 10, hexose translocator; 11, phosphate translocator; 12, ADP-glucose pyrophosphorylase; 13, starch synthase.

1988). The cytosolic form of PGI may thus be involved in converting the glucose moiety of the incoming sucrose to fructose 6-P according to the scheme given above. Higher affinity of the enzyme for fructose 6-P will favour the latter's conversion to glucose 6-P, which could ultimately serve as the source of carbon for synthesis of starch in amyloplasts through conversion to glucose 1-P, which enters the amyloplast possibly through a hexose translocator (Keeling *et al.*, 1988; Tyson and ap Rees, 1988). Inhibition of the enzyme by 6-phosphogluconate, ribose 5-P and ribulose 5-P suggests a close relationship between the above set of reactions and the oxidative pentose phosphate pathway. Accumulation of intermediates of the oxidative pentose phosphate pathway will block the above set of reactions at the level of PGI. Once this route is blocked, the intermediates of the oxidative pentose phosphate pathway will be recycled for the production of fructose 6-P through the combined actions of ribose phosphate isomerase, ribulose phosphate 3-epimerase, transketolase and transaldolase, etc. Hence, after producing reducing power, the intermediates of this pathway may be utilized for providing energy and extra carbon for onward transport to plastids for the synthesis of starch. Thus, the pathway could play a role in regulating the proportion of carbon shuttled through glycolytic and gluconeogenic reactions.

Acknowledgement

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Cholesterol binding reserve of hyperlipemic rat serum lipoproteins in chronic ethanol administration

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Abstract. Chronic administration of ethanol in rats caused the reduction of serum cholesterol binding reserve. The very low density and high density lipoproteins, main serum cholesterol binding reserves, were slightly increased with corresponding increases in their lipid and protein components during initial stage of alcohol consumption. However, these capacities get deminished during reversal of hyperlipemia induced by prolonged action of ethanol. This situation may be an early indicator for the initiation of hepatic damage and a variety of secondary effects of ethanol.

Keywords. Serum cholesterol binding reserve; lipoprotein cholesterol binding reserve; hyperlipemic; ethanol administration; liver damage; lipoproteins in alcoholism; lipid metabolism.

Introduction

Hsia *et al.* (1975) have demonstrated that serum can solubilize a considerable amount of exogenously added cholesterol. This capacity of serum was termed as serum cholesterol binding reserve (SCBR) and it accounts for its potential to solubilize cholesterol from arterial walls through reverse cholesterol transport mechanism (Stein *et al.*, 1975). Borresen and Berg (1981a, b) reported that SCBR can be attributed to cholesterol solubilization capacity of two lipoprotein subfractions, one of which contains high density lipoprotein (HDL) as major component, and the other very low density lipoprotein (VLDL). Hence it should and does correlate well with clinical parameters of diseases related with disorders of lipid metabolism. SCBR has been shown to get diminished in myocardial infarction (Chandra *et al.*, 1982), diabetes mellitus (Ghatak *et al.*, 1985) atherosclerosis and hypertension (Chandra *et al.*, 1980) and nephrotic syndrome (Perez *et al.*, 1979). Chronic administration of ethanol has been known to cause marked alterations in lipid and lipoprotein metabolism (Chander *et al.*, 1987, 1988). However information on cholesterol binding reserve of lipoproteins in alcoholism and its relationship with lipoprotein lipids is not available in literature. Therefore, it was considered of interest to study the cholesterol binding reserve of rat serum lipoproteins at various stages of hyperlipemia induced by ethanol feeding and the results are presented in this communication.

CDRI Communication No. 4321.

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Abbreviations used: SCBR, Serum cholesterol binding reserve; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; CBR, cholesterol binding reserve; TC, total cholesterol; PL, phospholipid; TG, triglyceride.

Materials and methods

Male adult rats of Charles foster strain (150–200 g) inbred in CDRI animal house were divided into control and alcohol fed groups of 8 rats in each group. Ethanol was administered in a 50% (v/v) aqueous solution (3·76 g/kg body weight) by gastric tubing once a day for 60 days. Control rats were given normal saline. At the end of the experiment representing 20, 30, 40, 50 or 60 days of alcohol treatment, the animals were fasted overnight, blood was withdrawn by retro orbital plexus and centrifuged. The serum was fractionated into VLDL, LDL and HDL by polyanionic precipitation methods (Burstein *et al.*, 1982) using heparin, dextran sulphate (molecular weight 5×10^5) and $MnCl_2$ as reactants. Each fraction was dialysed, freeze dried and quantitated according to the method of Hervie *et al.* (1973). Cholesterol binding reserve in serum lipoprotein was measured as described earlier by Hsia *et al.* (1975) with slight modifications. Control and hyperlipemic serum (0·5 ml) and lipoproteins (4 mg) dissolved in normal saline were mixed with 7 mg of cholesterol sonicated to a particle size of 10–60 μm and incubated at 37°C in a metabolic shaker with 60 strokes per min for 16 h. After incubation, the undissolved cholesterol was removed by filtering through Whatman No. 42 filter paper and cholesterol estimation in control and experimental sets with or without exogenous cholesterol were carried out by the method of Zlatkis *et al.* (1953). Cholesterol binding reserve was calculated as the difference between the two determinations and expressed in mg per 100 ml, as described by Perez *et al.* (1979). Lipoprotein and serum samples were delipidated with ethanol : ether (3 : 1 v/v). The apoprotein content was estimated by the method of Radding and Steinberg (1960) and Lowry *et al.* (1951). The lipid extracts were used for the estimation of total cholesterol (TC), phospholipid (PL) (Wagner *et al.*, 1961) and triglyceride (TG) (Handel and Zilversmit, 1957).

Results

Chronic administration of ethanol to rats resulted in increased level of SCBR which was maximum during 30 days of treatment (table 1). However, on further feeding of

Table 1. Cholesterol binding reserve in hyperlipemic rat serum lipoproteins.

Period of treatment (days)	Experimental schedule	Serum	VLDL	LDL	HDL
20	Control	33·63 ± 2·60	10·98 ± 1·00	0·156 ± 0·010	17·68 ± 1·78
	Ethanol fed	39·52 ± 3·04[a]	13·89 ± 1·44	0·157 ± 0·014[b]	20·14 ± 1·29
30	Control	34·61 ± 2·20	11·81 ± 1·01	0·151 ± 0·010	17·10 ± 1·35
	Ethanol fed	40·74 ± 3·36	14·34 ± 2·07	0·159 ± 0·018[b]	20·76 ± 2·39
40	Control	35·71 ± 2·28	11·62 ± 1·21	0·156 ± 0·020	18·75 ± 1·70
	Ethanol fed	39·01 ± 2·10[a]	13·19 ± 0·68[a]	0·156 ± 0·014[b]	19·49 ± 1·10[b]
50	Control	36·64 ± 2·33	11·70 ± 1·34	0·159 ± 0·014	17·39 ± 1·25
	Ethanol fed	33·47 ± 1·40[a]	10·79 ± 0·68[a]	0·160 ± 0·022[b]	16·38 ± 1·20
60	Control	36·07 ± 2·66	11·75 ± 1·55	0·154 ± 0·020	19·06 ± 1·57
	Ethanol fed	29·94 ± 2·70	9·61 ± 0·90[a]	0·150 ± 0·013[b]	14·25 ± 2·00

Values are expressed as mg/dl ± SD of 6 separate observations.
$P<0{\cdot}01$; [a]$P<0{\cdot}05$; [b]P not significant.

ethanol to these animals for 60 days, the levels were observed to decrease by 17% as compared to control. It may be seen that CBR of lipoproteins in control rats did not show any perceptible change during the experimental period of 60 days. The cholesterol binding was maximum in case of HDL, being 1·5-fold more than in VLDL. The levels of CBR of HDL and VLDL as a function of time, were comparable with those of SCBR during the course of treatment and were observed to decrease by 24·40 and 18·20% respectively as compared to control during 60 days of ethanol treatment. LDL of control as well as hyperlipemic rat serum failed to demonstrate the CBR capacity to any significant extent (table 1). It may be seen (figures 1–4) that the levels of serum components *viz.* TC, PL, TG and protein exhibited a progressive increase with marked increase in TG content up to 40 days of ethanol treatment followed by a fall during the subsequent period of treatment. The contents of TC, PL, TG and apoprotein of VLDL, LDL and HDL increased to varying extents, as a result of chronic ethanol intake (figures 2–4). However, the patterns of these alterations as a function of time were similar to those of serum lipids and protein. The VLDL from these animals was found to have 2-fold more TG than the controls. The parameter which shows unique behaviour under the chronic administration of ethanol is the CBR values of serum HDL and VLDL but not of LDL. After attaining its peak value on the 30th day of alcohol treatment it starts declining progressively and becomes much lower than the normal value on the 60th day of treatment.

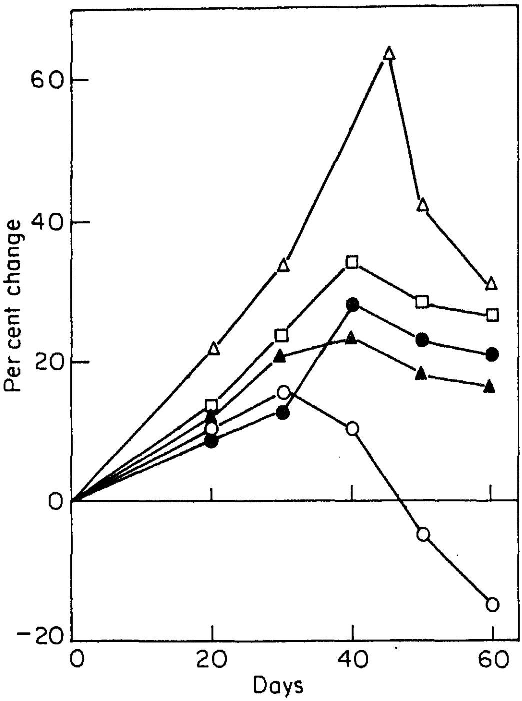

Figure 1. Effect of alcohol feeding on the CBR, lipid and protein contents of hyperlipemic rat serum. (○), SCBR (35·33 ± 1·2); (□), TC (97·3 ± 1·5); (▲), PL (68·75 ± 0·94); (△), TG (77·26 ± 2·74); (●), protein (5670 ± 170). Control values (in mg/dl) obtained from 6

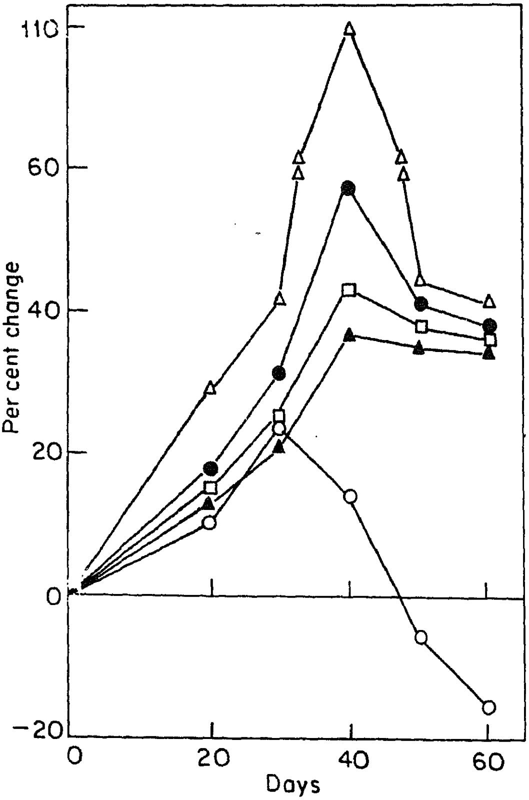

Figure 2. Effect of alcohol feeding on the CBR, lipid and protein contents of rat serum VLDL. (○), CBR (11·27 ± 0·33); (□), TC (4·44 ± 0·06); (▲), PL (5·87 ± 0·28); (△), TG (34·12 ± 0·85); (●), apoprotein (4·35 ± 0·23). Control values (in mg/dl) obtained from 6 rats are given as mean ± SD in parantheses.

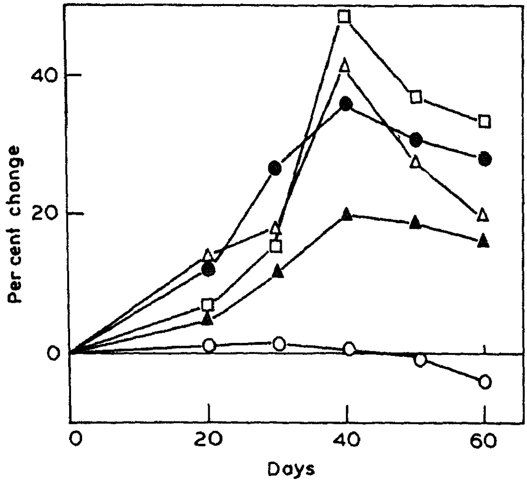

Figure 3. Effect of alcohol feeding on CBR, lipid and protein contents of rat serum LDL. (○), CBR (0·155 ± 0·012); (□), TC (8·9 ± 0·32); (▲), PL (7·63 ± 0·17); (△), TG (16·72 ± 0·10); (●), apoprotein (14·94 ± 0·24). Control values (in mg/dl) obtained from 6 rats are given as mean ± SD in parantheses.

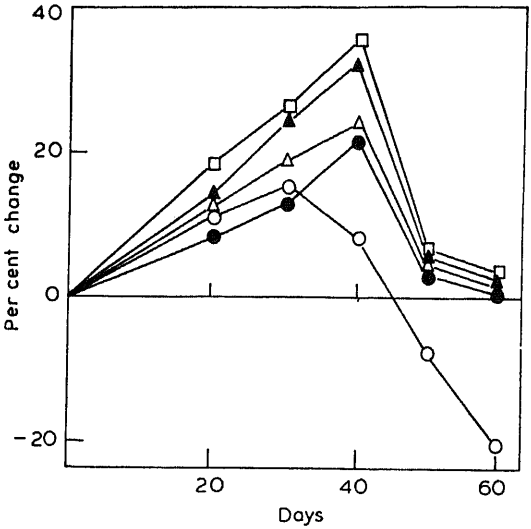

Figure 4. Effect of alcohol feeding on CBR, lipid and protein contents of rat serum HDL. (○), CBR (17·99 ± 0·86); (□), TC (38·35 ± 0·42); (▲), PL (27·25 ± 1·05); (△), TG (15·90 ± 0·25); (●), apoprotein (151·16 ± 2·72). Control values (in mg/dl) obtained from 6 rats are given as mean ± SD in parantheses.

Figures 1–4, show that SCBR and CBR of lipoproteins increase with correspond- ; increase in their lipids and proteins up to 30 days of treatment. However, these pacities tend to decrease progressively afterwards and do not manifest any ationship with the alterations in the levels of their lipid or protein components der the action of ethanol for 60 days. LDL has least binding capacity in both ntrol as well as in ethanol treated groups, although TC (8·9 ± 0·32 mg/dl), PL 53 ± 0·17 mg/dl), TG (16·70 ± 0·20 mg/dl), and apoprotein (14·94 ± 0·25 mg/dl) re increased by about 39, 18, 34 and 36% respectively at peak period of hyper- emia caused by ethanol treatment.

scussion

e present study demonstrates the alteration in CBR of serum and its lipoproteins LDL and HDL), which in turn are associated with alterations in their lipid and otein components under the action of ethanol. Increased CBR during early atment with ethanol is likely to enable the serum lipoproteins to remove lipids m tissues of hyperlipemic subject (Kramsch and Hollandu, 1973; Malinow, 1981). e increased production of HDL plays a compensatory role in counteracting fat cumulation with enhanced CBR activity in alcohol fed rats (Aleindor *et al.*, 1970). creased levels of SCBR and serum HDL cholesterol have been reported in *Curum oticum* (Ajawan) treated rabbits (Agrawala and Pant, 1987). The present findings present the reversal of hyperlipemia after 40 days along with lowering of oprotein lipids and protein levels leading to decreased function of cholesterol nding reserve. The abnormal levels of CBR may indicate the initiation of risk tors manifestable with hyperlipemia followed by transition from fatty liver to re advanced lesions and damage. Borrowsky *et al.* (1976) reported that the release of liver fat into blood in the initial stage is followed by a relative failure of fat removal from the liver at the advanced stage of hyperlipemia induced by ethanol in baboons which may cause liver damage. Perez *et al.* (1979) revealed that SCBR in normal and patients with nephrotic syndrome tend to increase with increasing serum triglyceride values. However, these levels in normal subjects increase with increase in cholesterol, while the SCBR shows no increase in the nephrotic group. Borresen and Berg (1981a, b) have studied the effect of lipid lowering drug 'Gemsfibrozil' in hyperlipemic humans and found a significant increase in the levels of HDL apoprotein, Apo A_I, Apo A_{II} and SCBR. Miller (1980) has shown that the binding capacities are directly related with surface activity of the intrinsic protein components constituting apoproteins of VLDL and HDL, and any alteration in the levels of these active proteins may influence the CBR of their lipoproteins. The abnormally unique response of CBR after 30 days of ethanol treatment may be due to serious alterations of the protein component (apoprotein) of the lipoproteins. The patients with history of chronic alcoholism have been shown to have structural and functional changes in lipoprotein patterns accompanying hepatic damage (Weidman *et al.*, 1982). CBR of LDL was not affected by ethanol feeding which would suggest that LDL apoprotein fails to contribute towards the binding properties of exogenously added cholesterol. The decline in CBR of lipoproteins (VLDL and HDL) during prolonged alcohol intake may be due to the toxic affects of lipid peroxides and free radicals produced in alcoholism. This is supported by our previous finding on lipid peroxidation of serum lipoproteins in chronic alcohol administration (Chander *et al.*, 1988). The diminished SCBR would be a sensitive marker of hyperbetalipoproteinemia and hepatic damage in alcoholism.

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anges in γ-aminobutyric acid during different stages of picrotoxin-uced seizure, and the effect of pretreatment with γ-acetylenic GABA d phenobarbital

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Abstract. Changes in GABA content of various brain areas during different stages of picrotoxin-induced seizures and following pretreatment with the anti-convulsants phenobarbital and γ-acetylenic GABA were studied. Picrotoxin (6 mg/kg) produced clonic/tonic convulsions associated with a 34% reduction in GABA content of the sensory motor cortex. A reduction of 24% was observed 1 min before the onset of seizure and the reduction in GABA content was reversible 20 min after the convulsion. No significant changes were observed in the cerebellum or spinal cord/medulla oblongata. Pretreatment with phenobarbital (100 mg/kg) delayed the onset of convulsion and decreased the mortality rate without causing any change in GABA content at the pre-convulsive, convulsive or post-convulsive stages. γ-Acetylenic GABA (100 mg/kg) has elevated GABA levels in different areas of the brain by 2–3-fold after 60 min treatment. This increase was reduced by 44% during the onset of picrotoxin-induced seizures. Picrotoxin convulsion can occur in the presence of normal, reduced or even elevated brain GABA content. The only consistent factor is a one-third reduction in GABA content before the onset of seizure.

Keywords. GABA; γ-acetylenic GABA; picrotoxin; phenobarbital.

roduction

s now generally accepted that γ-aminobutyric acid (GABA) is one of the main ibitory transmitters in the mammalian central nervous system (Curtis and nston, 1974; Krnjevic, 1974) and to some extent there is a correlation between in GABA content and its neuronal activity. Thus drugs which reduce brain BA content are often convulsants (Killam and Bain, 1957; Bradford, 1976). ses of reduced GABA content were reported after afferent electrical stimulation he brachial plexus (Lichtshtein and Dobkin, 1976), high pressure oxygen induced vulsions (Wood *et al.*, 1967; De-Feudis and Elliot, 1968), thiosemicarbazide-uced seizures (Wood *et al.*, 1966) and following picrotoxin-induced seizures dul-Ghani, 1980).

On the other hand, all inhibitors of GABA transaminase, which elevates brain BA content, have anticonvulsive properties (Collins, 1973; Jung *et al.*, 1977; echter *et al.*, 1979; Loscher and Vetter, 1984). Augmentation of GABA trans-ssion could also be brought about by blocking reuptake into presynaptic mem-nes and glia (Krogsgaard-Larsen *et al.*, 1981; Schousboe *et al.*, 1983; Wood and ddes, 1983). Previous results in our laboratory have shown the protective effect γ-acetylenic GABA and phenobarbital against picrotoxin-induced convulsion dul-Ghani *et al.*, 1987).

The aim of the present investigation is to test the pattern of change in GABA tent of different areas of the brain during the development of picrotoxin vulsion, and the effect of pretreatment with phenobarbital and γ-acetylenic BA.

Materials and methods

Materials

γ-Acetylenic GABA [4-amino hex-5-ynoic acid, RMI (71645)] was a gift of Dr J. Wilkins and his colleagues at the Centre de Recherche Merrell International, Strasbourg, France.

Picrotoxin, phenobarbital and gabase were purchased from the Sigma Chemical Co., St Louis, Missouri, USA.

Methods

White female albino mice weighing 25–30 g were used in the experiments. The animals were allowed free access to food and water before treatment. The mice were distributed into 3 groups and were injected intraperitoneally either with phenobarbital (100 mg/kg) or with γ-acetylenic GABA (100 mg/kg) 45–60 min before injection of the convulsant picrotoxin (6 mg/kg). Control animals were pretreated with the same volume of 0·9% (w/v) NaCl.

After injection of picrotoxin the mice were under constant observation for measuring the time to onset of convulsion and the mortality rate. At different pre-convulsive, convulsive and post-convulsive stages, animals were stunned and killed by cervical dislocation, and the brains were rapidly removed and frozen on dry ice. The brains were dissected into 3 main areas, sensory motor cortex, cerebellum and spinal cord/medulla oblongata combined. Each part was quickly weighed and dropped into ice-cold trichloroacetic acid 10% for homogenization and centrifugation. The supernatants were stored frozen (−70°C) until assay for GABA content.

GABA content was measured using the enzymatic method. Gabase (Sigma) contains both GABAtransaminase (5-aminobutyrate; 2-oxoglutarate aminotransferase; EC 2·6·1·19) and succinate semialdehyde dehydrogenase [succinate semialdehyde: NAD(P) oxidoreductase; EC 1·2·1·16]. The reaction mixture consisted of 0·1 M potassium pyrophosphate pH 8·6, 0·004 M NADP pH 7, 0·02 M α-ketoglutarate solution pH 7·9 and 0·2 units/ml gabase. Extract equivalent to 25 mg of brain tissue was added to 3 ml of reaction mixture.

Concentrations of GABA are expressed in this paper as μmol per gram of fresh tissue. Where required, statistical analysis was performed using Student's '*t*' test.

Results

Convulsive action of picrotoxin

Injection of mice with picrotoxin (6 mg/kg) produced clonic convulsions after a latent period of 8–11 min. These convulsive movements gradually increased and ended in a tonic extensor spasm of the 4 limbs after 12–15 min. Most of the animals died in this phase, and the remainder lay in a flaccid state until the second phase began—a clonic seizure less violent, less severe and of shorter duration. After 3–6 phases of clonic convulsion the convulsive activity vanished

We have found a relatively large decrease of 20% in GABA brain content associated with picrotoxin-induced seizure. At the onset of convulsion the concentration of GABA in the sensory motor cortex was significantly decreased (by 34%, from 1·64 ± 0·06 μmol/g tissue to 1·09 ± 0·10 μmol/g tissue, $P < 0{\cdot}01$). No significant changes were observed in the cerebellum or spinal cord/medulla oblongata (table 1).

Table 1. Distribution of GABA in different areas of the brain following treatment with picrotoxin, phenobarbital and γ-acetylenic GABA.

	Sensory motor cortex	Cerebellum	Spinal cord/ medulla oblongata
Control	1·64 ± 0·06 (20)	1·64 ± 0·13 (10)	1·66 ± 0·16 (10)
Picrotoxin convulsion	1·09 ± 0·10* (5)	1·35 ± 0·09 (5)	1·51 ± 0·16 (5)
Phenobarbital	1·59 ± 0·08 (11)	1·60 ± 0·06 (6)	1·65 ± 0·13 (6)
γ-Acetylenic GABA	3·59 ± 0·31** (18)	4·40 ± 0·35** (6)	3·10 ± 0·23* (6)

GABA content was measured in different areas of the brain of mice, following i.p. injection of picrotoxin (6 mg/kg), at the onset of seizure, or 60 min after the injection of phenobarbital (100 mg/kg) or γ-acetylenic GABA (100 mg/kg). The values are mean ± SEM for the number of experiments indicated in parentheses. GABA content expressed as μmol/g tissue.
*Significance of difference: $P < 0{\cdot}01$; **$P < 0{\cdot}001$, compared to control values.

One min before the onset of convulsion GABA level was reduced significantly (by 24%, to 1·25 ± 0·11 μmol/g, $P < 0{\cdot}025$). In 6 animals which recovered from convulsion GABA content of the sensory motor cortex increased to 1·45 ± 0·08 μmol/g 20 min after the onset of seizure (table 2).

Table 2. Effect of picrotoxin on GABA content of sensory motor cortex of mice pretreated with phenobarbital and γ-acetylenic GABA.

	Pretreatment		
	Saline	γ-Acetylenic GABA	Phenobarbital
Control	1·64 ± 0·06 (20) 100%	3·59 ± 0·31 (18) 100%	1·59 ± 0·08 (11) 100%
Pre-convulsive stage	1·25 ± 0·11 (8) 76% $P < 0{\cdot}025$	2·19 ± 0·14 (9) 61% $P < 0{\cdot}005$	1·46 ± 0·05 (5) 92% NS
Convulsive stage	1·09 ± 0·10 (5) 66% $P < 0{\cdot}01$	2·01 ± 0·11 (10) 56% $P < 0{\cdot}005$	1·54 ± 0·04 (8) 97% NS
Post-convulsive stage	1·45 ± 0·08 (6) 88% NS	2·85 ± 0·32 (6) 79% NS	1·58 ± 0·06 (7) 99% NS

The values are mean ± SEM for the number of experiments indicated in parentheses, GABA content expressed as μmol/g tissue.
NS, Not significant.

Anti-convulsive action of phenobarbital

Although phenobarbital (100 mg/kg) delayed the onset of picrotoxin convulsion, did not change GABA content of the whole brain or of any of the tested areas. Similar results were observed during pre-convulsive, convulsive and post-convulsive stages (table 2).

Anti-convulsive action of γ-acetylenic GABA

γ-Acetylenic GABA (100 mg/kg) increased GABA levels in the whole brain by 2–3-fold. The maximum effect was seen in the cortical area; the effect was slightly lower in the spinal cord/medulla oblongata (table 1).

Intraperitoneal injection of picrotoxin (6 mg/kg) to mice pretreated 45 min earlier with γ-acetylenic GABA produced a maximum reduction in GABA content of 44% ($P < 0{\cdot}005$) at the onset of seizure (table 2). One min before the onset of convulsion GABA content was clearly decreased by 39% and in the post-convulsive stage the effect was partially reversed (~20%, not significant).

Discussion

Picrotoxin, a potent convulsant which acts by blocking the inhibitory synaptic action of GABA, has been reported to produce a strong clonic/tonic convulsion with a high mortality rate and a significant reduction in GABA content of brain at the onset of convulsion (Loscher and Frey, 1977; Abdul-Ghani *et al.*, 1987).

The fact that the reduction in brain GABA content was localized to the cortical area, with no significant decrease in GABA content of cerebellum or spinal cord/medulla oblongata (table 2), is of special interest since picrotoxin acts as an antagonist of GABA inhibitory action in different areas of the nervous system including brain stem (Straughan, 1974), spinal cord (Engberg and Thaller, 1970), cerebral cortex (Hill *et al.*, 1972) and cerebellar cortex (Bisti *et al.*, 1971).

The present report shows that γ-acetylenic GABA produced an increase in GABA content of 2–3-fold; the amount of increase differing among various brain regions, with the greatest change in cortical areas. The picrotoxin convulsion occurred in mice pretreated with γ-acetylenic GABA, even with relatively high levels of GABA in these animals. However, the convulsions occurred after a 44% decrease in GABA content in the pretreated animals. The decrease in GABA content was seen before the onset of convulsion, and was partially reversed in the post-convulsive stage. These results would explain the slight anti-convulsant properties of γ-acetylenic GABA with regard to picrotoxin seizures. Similar anti-convulsive activity was found against strychnine- and thiosemicarbazide-induced seizures (Schechter *et al.*, 1977).

Although phenobarbital has shown the best anti-convulsive activity (Abdul-Ghani *et al.*, 1987) it had no effect on brain GABA content regardless of the area tested. This was true in pre-convulsive, convulsive and post-convulsive stages (table 2). Previous results have shown no correlation between the anti-convulsive activity of phenobarbital and changes in extracellular GABA accumulation (Iversen and Johnston, 1971; Abdul-Ghani *et al.*, 1981). It is more likely that the anti-convulsive action of phenobarbital is due to its effect on the GABA receptor complex.

concentrations. The only consistent factor is a one-third reduction in GABA content before the onset of seizure. This becomes important in the light of the fact that the neuronal pool of GABA is approximately one-third of the total brain GABA content (Patel *et al.*, 1974; Sellstrom *et al.*, 1975).

On the other hand the protective action of anti-convulsants is produced by those which raise seizure threshold, either by elevating brain GABA level or by reducing brain aspartate or glutamate levels. Other investigators have shown that correlation does exist between the increase in synaptosomal GABA levels and the delay in the onset of seizures (Wood and Geddes, 1983).

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at ventral prostate as an independent source of inhibin-like material

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Abstract. Castration had no effect on prostatic inhibin-like activity as compared to normal controls. Consequent upon castration there is a significant reduction in the inhibin content of the ventral prostate on a per gland basis. However, when expressed as a function of protein content, there is no change. The data suggest that the very same epithelial cells which under the influence of androgen carry out the characteristic exocrine secretory activity are responsible for the synthesis and secretion of inhibin-like material.

Keywords. Rat ventral prostate; inhibin-like activity; inhibin content; epithelial cells.

troduction

ı exciting recent development in endocrinology is the realization that regulatory
ptides occur in sites other than their 'classical' sites of synthesis (Niall, 1981;
kary *et al.*, 1982; Bigazzi and Bryant-Greenwood, 1984; Dufy-Barbe, 1985). The
st indication of the presence of inhibin in the human prostate was obtained by
ıze *et al.* (1979) in the course of their efforts to develop a radioimmunoassay
IA) for inhibin. Human prostate exhibited the highest content of radioimmuno-
sayable inhibin-like material (ILM) as compared to the seminal vesicle, the
ididymis or the testis (Vaze *et al.*, 1980). Subsequently Sheth *et al.* (1981) isolated
d partially purified biologically active as well as immunoreactive inhibin from the
man prostate. In a comparative study of human testes, prostate, and seminal
ısma as sources of inhibin, Krishnan *et al.* (1982) found that irrespective of the
urce, inhibin bio-activity showed the same elution characteristic on Sephadex G-
0 columns. These authors claimed that testicular, prostatic and seminal plasma
ıibin are indistinguishable physicochemically, biologically and immunologically
ıeth *et al.*, 1981). This, taken together with the observation of Asch *et al.* (1980)
the presence of inhibin in the seminal plasma of vasectomized subjects, led Sheth
al. (1981) to hypothesize that the prostate concentrates circulating inhibin and
eases it as a constituent of its secretion into the seminal plasma. In support of
ch a view Phadke *et al.* (1982) and Vanage *et al.* (1985) claimed the presence of
gh affinity inhibin receptors in human and rat prostate respectively.

The alternative view that the prostate could be an extratesticular site of synthesis
s also received support from Sheth *et al.* (1981). These workers have reported that
ε prostatic tissue of castrated individuals contains ILA, although its concentration
lower than that of normal subjects. They also observed a 10-fold increase in
ıibin content in cases of benign prostatic hyperplasia (BPH) as compared to

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o whom all correspondence should be addressed.

breviations used: RIA, Radioimmunoassay; ILM, inhibin-like material; ILA, inhibin-like activity;
'H, benign prostatic hyperplasia; hCG, human chorionic gonadotropin; FSH, follicle stimulating
rmone; hMG, human menopausal gonadotropin.

normal tissue. The high level of inhibin in BPH tissue was also reflected in the serum (Sheth *et al.*, 1982).

The following observations add weight to the concept that the prostate is an extragonadal source of ILM: (i) the presence of a higher proportion of ILM in that fraction of the split ejaculate that is known to be rich in prostatic secretion (Beksac *et al.*, 1984), (ii) a high level of ILM in the serum, semen and urine of gonadectomized men (Beksac *et al.*, 1984) and (iii) the further enhancement of postcastrational serum follicle stimulating hormone (FSH) levels following prostatectomy (Sheth *et al.*, 1984).

Kharbanda *et al.* (1985) demonstrated unambiguously that the rat ventral prostate is indeed an independent source of inhibin in as much as ILA persisted despite epididymectomy/castration. The present work was undertaken to further substantiate the androgen independent secretion of prostatic inhibin.

Materials and methods

Animals

Holtzman derived male albino rats from our inbred animal colony maintained under standard conditions (temperature: 24 ± 1°C; 14 h light: 10 h dark) were used for the entire study. They were fed adlibitum on a commercial balanced pelletized diet. Tap water was freely available. The age of the animal was reckoned starting from day 0, the day the litters were born. All rats were weaned at day 21.

Orchidectomy

Two months old adult male rats were bilaterally castrated *via* the scrotal approach. These animals were autopsied 6 weeks after surgery along with appropriate age-matched normal controls. The ventral prostate was removed, freed of adhering connective tissue, weighed on a torsion balance and processed in one of two alternative ways: (i) an acetone powder was prepared and assayed for ILA by the 'Moudgal assay' as detailed earlier (Kharbanda *et al.*, 1985) and (ii) the tissue was homogenised in saline (10% w/v), centrifuged and supernatant recovered. Protein in the supernatant was determined by Layne's (1957) procedure.

Histology

Four days as well as two and six weeks after surgery, the ventral prostate was removed, freed of adhering connective tissue and fixed in Bouin's overnight. After fixation, the tissue was washed, dehydrated through grades of alcohol, cleared in benzene and infiltrated with wax. Blocks were made and serial sections cut at 5 μm. The sections were stained with hematoxylin (Sigma) and eosin (BDH).

Statistics

The data was statistically analysed by two-way analysis of variance (ANOVA). For the various parameters measured, the magnitude of correlation was established using the standard correlation analysis and wherever warranted, regression analysis was conducted (Sokal and Rohlf, 1969). To test for difference between means, after significant ANOVA main effects, the student's Newman-Keul's post-hoc test was used. A P value $<0{\cdot}05$ was considered statistically significant. The level of significance of difference from human chorionic gonadotropin (hCG) control is denoted by asterisks.

Slope values of the dose-response lines and indices of precision were determined using Borth's analysis of multiple parallel line assays based on a symmetrical design (Borth, 1960). In our assay, we have used two doses with a log dose-interval of 2, with 4 mice per dose. The level of significance was set at 5%. If parallelism in the slope of the log dose-responses was observed, the data was used for calculating relative potency according to Ramasharma *et al.* (1979).

Results

Observations on ILA

Castration had no effect on the prostatic ILA as compared to normal control ($P>0{\cdot}1$, F-test; figure 1).

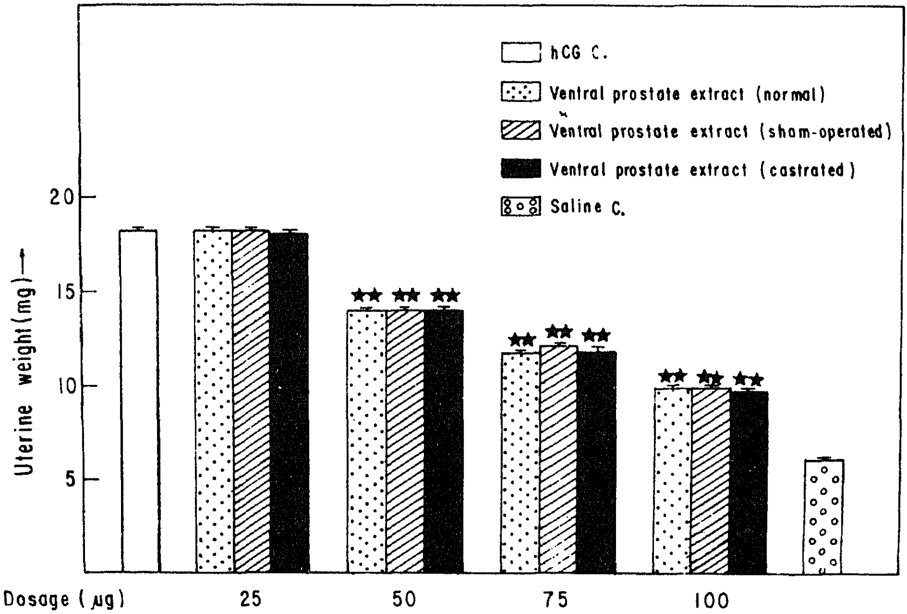


Figure 1. Effect of castration on production of rat prostate inhibin.

The bar chart represents the mean ± SEM, n = 4.

Castration does not affect the prostatic inhibin activity compared to Sham-operated or age-matched normal controls. ($P>0{\cdot}1$). A dose-dependent suppression of uterine weight gain is observed compared to hCG-control ($P<0{\cdot}001$) when saline extracts of ventral prostate acetone powder of normal, Sham-operated or castrated rats is administered to the test animals.

Statistical evaluation of the assay procedure based on Borth's system of computation (Borth, 1960) is summarized in table 1.

Table 1. Statistical evaluation of the 'Moudgal assay' by Borth's system of computation.

	Parameter										
	$\bar{n}$	J	I	b_c	S_c	S_c^2	V	g_c^*	G_c^2	$F_v^\dagger$	λ^{**}
Value	4	2	0·301	113·52	1·62	2·623	0·656	0·003	0·691	1·054	0·014

$\bar{n}$, Average number of animals per dose level; J, ratio of contiguous doses; I, $\log J$; b_c, combined slope; S_c, standard deviation of response; S_c^2, residual variance; V, variance of the mean effects; G_c, index of significance of slope; G_c^2, departure from parallelism; F_v, F value for departure from parallelism; λ, index of precision.

*The computed value of g_c is <1 and therefore the combined slope for the assay can be considered significant.

†The computed value of F_v is less than the table value of 4·75. Therefore departure from parallelism is negligible.

**The value of λ is $<0{\cdot}2$ and therefore the index of precision of the bioassay is acceptable.

The data for linear regression when plotted (figure 2) show identity of the dose-dependent response for both preparations. Using the data for calculating potencies, as defined by Ramasharma *et al.* (1979), it is seen that 50% suppression of the hCG induced uterine weight increase in treated mice is effected by 68 μg of either preparation. This is ofcourse in line with the observation that the two preparations give an identical log dose-response curve. The inhibin content (expressed as units/mg tissue protein or units/gland) was then calculated and the potency compared (table 2). Consequent upon castration, there is a significant reduction in the inhibin content of the ventral prostate on a per gland basis. However, when expressed as a function of protein content, there is no change.

Histological observations

The histology of the normal rat ventral prostate is shown in figures 3 and 4. Castration-induced regression is evident within 4 days of surgery (figure 5). Consequent upon involution of the gland, the typical architecture of the acini is lost. Another characteristic effect is that the height of the columnar epithelial cells is reduced. With prolonged effects of castration (figures 6, 7) the acini shrink further so that the basket-like investment of the smooth muscle cells becomes more evident. With progressive decrease in the volume of the columnar cells as well as their number, the stromal cells become increasingly prominent. The basal cells too are easily discernible in sections of ventral prostate of castrated rats (figures 5–7).

Discussion

The rationale behind the coupled assay as a convenient and rapid method of diagnosis and quantitation of ILA has been discussed (Kharbanda *et al.*, 1985, Chari

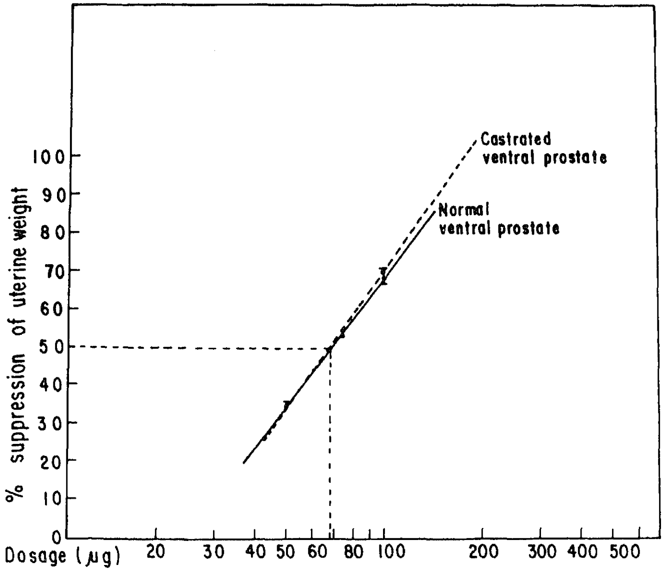


Figure 2. Log dose-response curves for inhibin activity for saline extracts of ventral prostate acetone powder of normal and castrated rats. The different preparations were tested in the 'Moudgal assay' at 3 dose levels. The results are expressed in terms of per cent suppression in uterine weight gain. This was calculated as follows:

$$\frac{\text{(Uterine weight of hCG control)} - \text{(Uterine weight of inhibin group)}}{\text{(Uterine weight of hCG control)} - \text{(Uterine weight of saline control)}}$$

Each value represents the mean ± SEM, n = 4.

Table 2.

	Normal ventral prostate	Castrated ventral prostate (6 week castrate)
n: number of animals	4	4
Wet weight (mg)	113·225 ± 16·26	14·35 ± 1·24
Protein content		
mg/gland	11·195 ± 0·447	1·422 ± 0·08
μg/mg wet weight tissue	104·349 ± 12·717	100·08 ± 4·555
μg for 50% suppression	68	68
Inhibin content		
Units/mg protein	14·705	14·705
Units/gland	164·632	20·912

, 1987) and validated using the *in vitro* pituitary cell culture assay (Dhingra Duraiswami, 1988). The results of the coupled assay proved unequivocally that atic ILA acts at the level of the pituitary (Kharbanda *et al.*, 1985). Therefore, ubsequent preparations were evaluated for ILA by the 'Moudgal assay' asharma *et al.*, 1979).

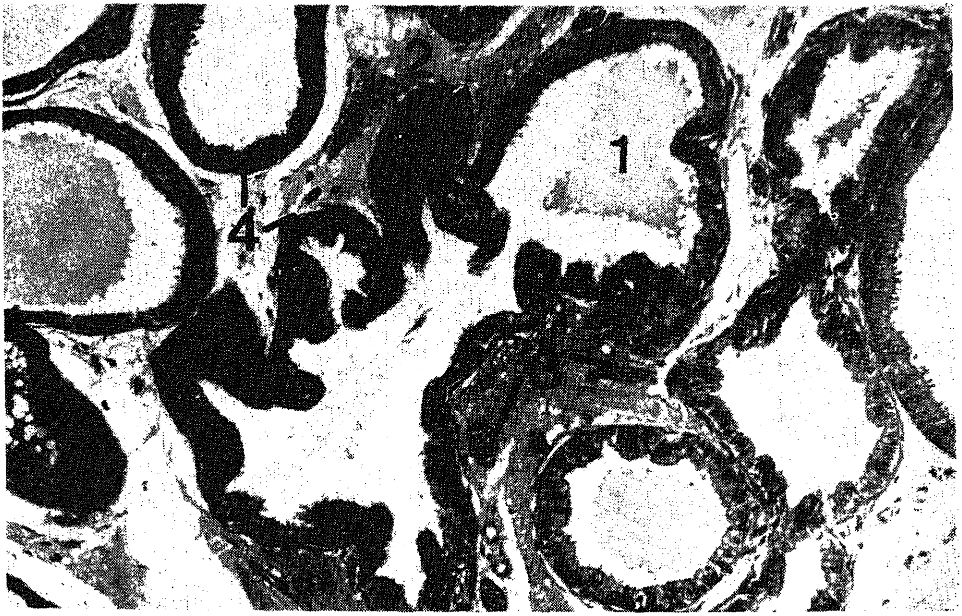
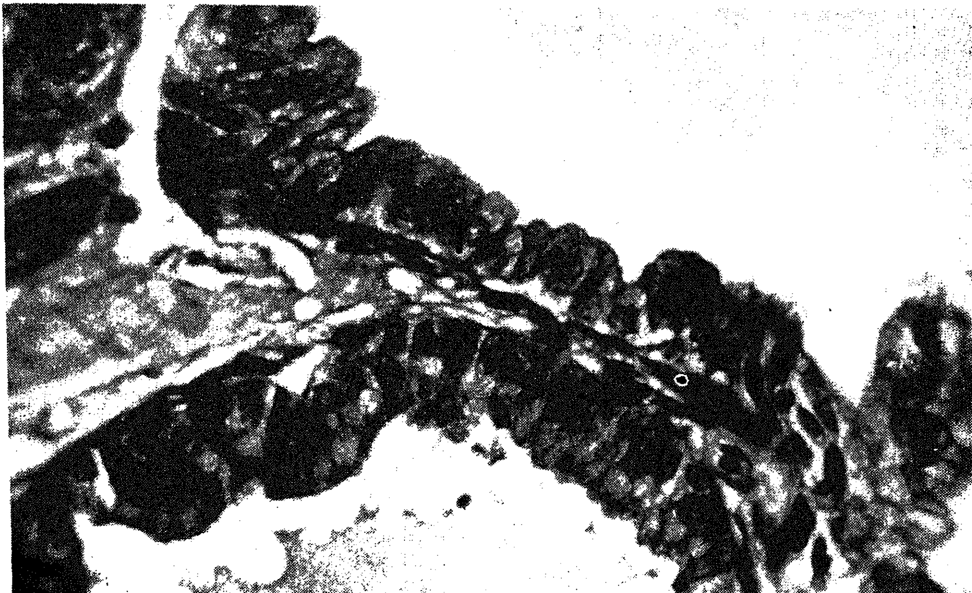


Figure 3. T.S. of normal rat ventral prostate. The gland exhibits large dilated acini containing secretory products in the lumina (1). Connective tissue (2) fills the space between the epithelial acini. Accumulation of interstitial tissue is seen at the intersection of several acini. Note the numerous blood vessels (3). The interstitial tissue is differentiated to form a sheath (4) around the acini. Hematoxylin and eosin staining (×250).



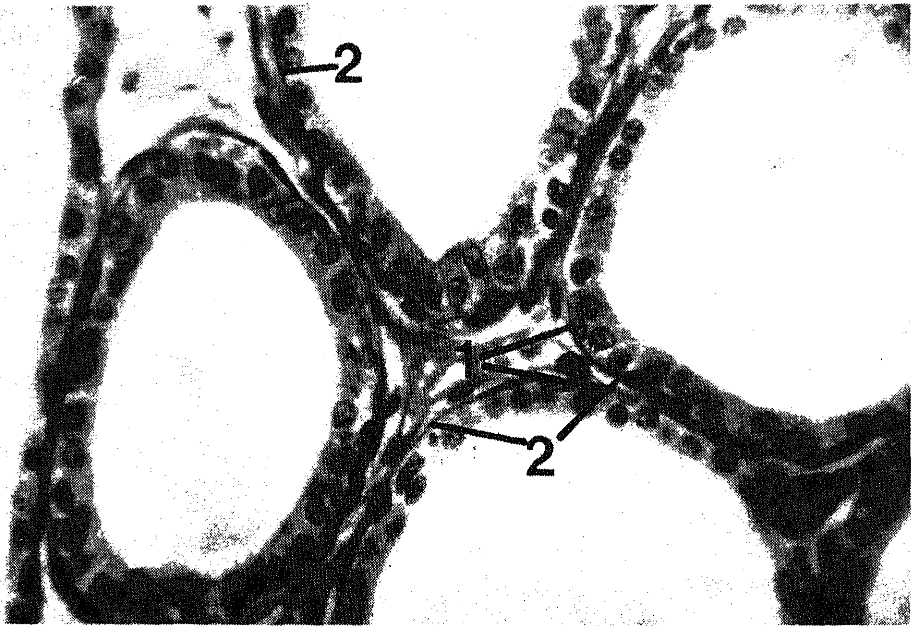


Figure 5. T.S. of rat ventral prostate 4 days after castration.
The diameters of acini are less than in intact animals. The infoldings of the glandular epithelium are lost. The height of columnar epithelial cells is reduced. The basal cells (1) of the glandular epithelium (with the long axes of their nuclei aligned parallel to the basement membrane) and the basket-like investment (2) of the smooth muscle cells is evident. Hematoxylin and eosin staining (× 800).

ollowing castration, two parameters show changes: (i) the wet weight of the nd and (ii) the inhibin content on a per gland basis. The histological changes cribed provide a basis for interpretation of the above results. It is clear that the state is indeed an androgen-dependent organ because, following castration, there a significant decrease in the degree of cellularity of the gland (Lesser and chovsky, 1973), mostly reflecting significant loss of the epithelial cells lining the ni (Rennie *et al.*, 1984). This would explain the decline in wet weight and the tein content per gland following castration. Since the inhibin content shows a line only on the basis of total glandular content, but not on a per mg protein is, two conclusions appear justified. Firstly, it is evident that it is the epithelial s that are the site of synthesis of prostatic inhibin. Had it been otherwise, *i.e.*, there been an involvement of the stromal or the basal cells in the synthesis of ibin, then the content per gland should remain unaffected by castration since se cells are not influenced by castration. Secondly, the synthesis of inhibin *per se* ndrogen-independent.

hen expressed as μg/mg wet weight of the tissue, the prostatic inhibin content s the same irrespective of whether the animals were castrated or not. This is in trast to most reports in the literature on protein content (Baulieu *et al.*, 1975; usette *et al.*, 1982). For example, Rajalakshmi and Prasad (1975) reported that tration decreased the incorporation *in vivo* of labelled amino acids into prostatic

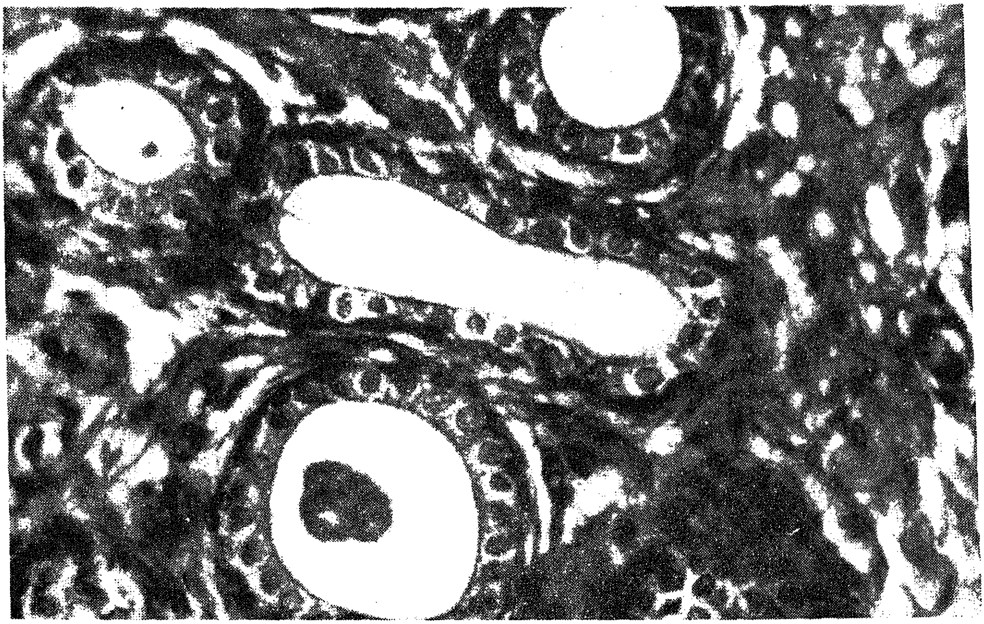


Figure 6. T.S. of rat ventral prostate two weeks after castration.

Histologically, the 2 weeks castrate differs from the normal principally in decreased acinar size and an increased prominence of the interacinar connective tissue (1). Hematoxylin and eosin staining (×800).

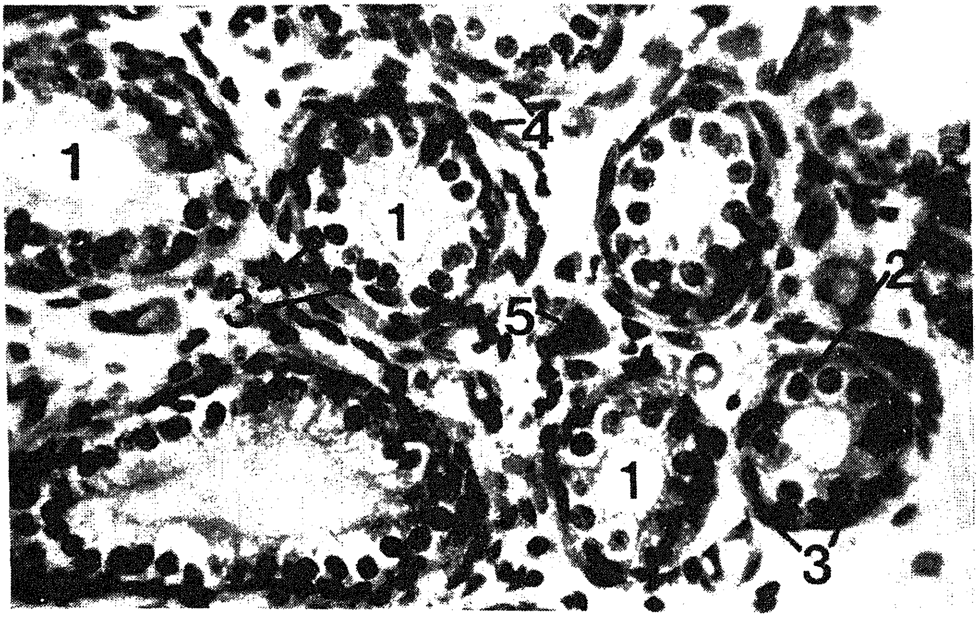


Figure 7. T.S. of rat ventral prostate 6 weeks after castration.

had no effect on the incorporation of [^{3}H]leucine into the total proteins of prostatic organ culture explants. Along the same lines, Dube *et al.* (1983) reported similar levels of incorporation of [^{3}H]leucine or [^{35}S]methionine into prostatic tissue slices from intact, castrated or steroid-treated animals, even though completely different sets of proteins were synthesized. These observations *in vitro* have been corroborated by the *in vivo* studies of Anderson *et al.* (1983) and Montpetit *et al.* (1986) showing that a substantial number of proteins were differentially induced or repressed by testosterone as evident from two-dimensional protein maps from normal, castrated and castrated + testosterone treated animals. Of the secretory proteins, some were clearly regulated in a highly differential manner but others appeared to be unaffected by castration.

Thus androgen deprivation does not lead to a total shut down of protein synthesis in the prostatic epithelial cells. However, synthesis of certain types of proteins, presumably androgen dependent, is shut off even as the synthesis of other proteins is switched on. Furthermore, the availability or otherwise of androgen appears to have no influence on the synthesis of certain proteins, one of them being inhibin.

It appears, therefore, that the prostate is an independent source of ILM thereby rendering it unlikely that prostatic inhibin is nothing but testicular inhibin concentrated by prostate from the general circulation. While inhibin synthesis *per se* is androgen independent, there is an indirect androgen influence in that, in the absence of androgen support, there is a significant reduction in the number of cells competent to produce inhibin.

Dias *et al.* (1983) have reported that the polyamines putrescine, spermidine and spermine inhibit the binding of [^{125}I]hFSH to receptors from calf testis. It may, therefore, be argued that the ILA demonstrated by the *in vivo* bioassay model is spurious and is to be attributed to the above polyamines of which the rat ventral prostate is a rich source (Mann, 1964; Pegg and Williams-Ashman, 1969; Williams-Ashman *et al.*, 1969; Williams-Ashman and Lochwood, 1970). Two considerations strongly militate against such a possibility. In the first place, the very manner of processing prostatic tissue, involving as it does precipitation of the tissue proteins with alcohol followed by repeated washings with alcohol and acetone, would eliminate these amines from the preparations being tested for inhibin activity. Moreover, while the effect of the polyamines is specifically on FSH-binding by gonadal receptors, in our assays saline extracts of rat ventral prostate acetone powder do not suppress uterine weight gain in the hMG-treated mice (Kharbanda *et al.*., 1985).

In light of the known presence of seminal plasmin as a significant constituent of seminal plasma (Thiel and Scheit, 1983), de Jong and Robertson (1985) have suggested that the dose-dependent suppression of hCG-induced uterine weight increase in mice injected with seminal plasma or fractions purified therefrom may well be due to the ability of seminal plasmin to suppress overall protein synthesis. This interesting suggestion, however, is hard to reconcile with our observation that such samples have an inhibitory effect only when the animals are treated with hCG but not with

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Validation of coupled bioassay for inhibin by pituitary cell culture assay

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Abstract. Studies on the characterization of inhibin and inhibin-like factors have depended for the most part on the classical *in vitro* pituitary cell culture assay. A major drawback with this assay is the turn-around time which is in the order of two weeks and consequently slows down purification efforts. The 24 h bioassay for inhibin has been found to be sufficiently sensitive and also statistically valid. Unfortunately, based as it is on a secondary response, ambiguities arise in interpreting the results. By including a parallel assay in which the mice are primed with human menopausal gonadotropin rather than human chorionic gonadotropin, it was possible to device the coupled bioassay. This enables distinguishing inhibin-like factors acting to suppress pituitary follicle stimulating hormone output from those acting at the level of gonads. In this study the coupled assay for inhibin has been compared with the classical pituitary cell culture assay in order to assess its biological and statistical validity. The data validates the bioassay on both the above counts and when considered in conjunction with the short turn-around time suggests that this assay can be highly useful in studies on isolation of inhibin from various sources.

Keywords. Inhibin; bioassay; specificity; statistics.

Introduction

The pioneering efforts of Baker *et al.* (1976) and de Jong *et al.* (1979) culminated in the adoption of the *in vitro* pituitary cell culture based assay as the method of choice for identifying and quantifying inhibin, the gonadal factor exerting feedback control on pituitary follicle stimulating hormone (FSH) output. The attractive features of this assay are its specificity, precision and sensitivity—factors that render it valuable in the clinical laboratory. However, a major drawback in its routine use in the research laboratory is the turn-around time which is of the order of 10–15 days. This militates against rapid progress in the fractionation and purification to homogeneity of inhibin/inhibin-like activity (ILA) from diverse sources—an objective still pursued in many laboratories around the world. Two other limitations that are serious enough for at least some laboratories are: (i) the cost and (ii) the hazard inherent to an assay dependent on the routine handling of radioactive iodine.

It would appear, therefore, that there is still a place for a simple, specific and rapid bioassay for inhibin that is at the same time economical. The bioassay developed by Ramasharma *et al.* (1979) appeared, at first sight to fulfill the above criteria. However, it was soon realized that it gave ambiguous results as instanced by experience in this laboratory in the course of characterizing bovine seminal plasma inhibin (Mohapatra *et al.*, 1985). In light of this and based on the observations of Lamond and Emmens (1959), a modified assay, the so called coupled bioassay with a turn-around time of 24 h, has been used for some years with great advantage in this laboratory (Kharbanda *et al.*, 1985; Chari *et al.*, 1987). In order to further document the biological and statistical validity of this assay, preparations showing either ILA or, in the alternative, FSH-BI or LH-RBI activity have been compared in this as well as in the classical pituitary cell culture assay (Dhingra and Duraiswami, 1988a). The data reported in the present study suggest that the coupled assay can be usefully employed in studies aimed at the isolation and characterization of inhibin/ILA from various sources.

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Materials and methods

Coupled assay

Swiss albino derived, colony bred, 24 ± 1 day old, immature female mice weighing 12 to 13 g were used. Choice of animals younger than those used by Ramasharma *et al.* (1979) was necessitated by the fact that in our colony vaginal opening occurred on day 27. They were housed under the standard conditions of the animal house (temperature 24 ± 1°C, 14 h light-10 h dark schedule, freely available commercial pelletted diet and water). The animals were randomly distributed to the various test groups and were assessed for uterine response to graded doses of test samples in two parallel assays, in one of which the control as well as treated animals received a total dose of 40 IU of human chorionic gonadotropin (hCG) (Pregnesine/Serono) while in the other they received a total dose of 5 IU of human menopausal gonadotropin (hMG) (Pergonal/Serono). Animals not receiving either gonadotropin but only saline at the scheduled times served as basal controls, while those receiving hCG or hMG and saline, in lieu of the test sample, served as gonadotropin-treated controls. The dose of hCG or hMG administered to the animals was such as to effect a tripling of uterine weight as compared to basal control. The injection schedule was as follows: Saline or test sample in appropriate dose was administered intraperitoneally at 09:00 h, followed 1 h later by hCG/hMG/saline *via* the subcutaneous route. The injections were repeated in the same order at 18:00 and 19:00 h. The total dose of each of the injectibles was divided into two equal halves and administered in a total volume of 0·4 ml., *i.e.*, 0·2 ml per injection. The animals were autopsied 15 h after the last injection. The uteri were dissected out, freed of adhering tissue and weighed to the nearest tenth of a mg in a torsion balance. In either case, the end point was a dose-dependent inhibition of gonadotropin (hCG/hMG) induced uterine weight gain.

Pituitary cell culture assay

All glassware used was siliconized and autoclaved. All fluids were sterilized by passing them through Millipore filters. Anterior pituitary glands were collected from adult male rats (Holtzman strain) after decapitation. Dispersed pituitary cells were prepared by the method of Vale *et al.* (1972) with the difference that hyaluronidase was omitted from the dispersion medium. Furthermore, the medium used for the final culture was M-199 (GIBCO) supplemented with 10% fetal calf serum, freshly added 0·1 mM glutamine and antibiotics. Approximately 5×10^5 cells were added to each well of a 24-well tissue culture treated polystyrene plate (Cell Wells, TM Corning) and cultured under 95% air, 5% CO_2 at 37 ± 1°C in 2 ml of culture medium. The assay itself was set up according to de Jong *et al.* (1979). The end point used was suppression of GnRH-stimulated FSH release.

Radioimmunoassays for gonadotropins were performed by the double antibody method (Midgley, 1966) using the NIAMDD rat RIA kits. The results are expressed with reference to NIAMDD-FSH-RP-1 (sensitivity, 4 ng) and NIAMDD-LH-RP-2 (sensitivity, 5 ng). Intra and interassay variations were respectively, FSH: 5 to 6% and 8 to 9%; LH: 6 to 7% and 10 to 12%.

Test preparations: Details of the samples used as test preparations in this study are provided in table 1.

Table 1. Test preparations.

Sample	Description	Biological property	Reference
oTI	Ovine testicular inhibin	Inhibin	Dhingra and Duraiswami (1988b)
oFFI	Ovine follicular fluid inhibin	Inhibin	Narendar Kumar (1985)
bSPI-DE2	Preparation DE-2 from bull seminal plasma	Inhibin-like	Malhotra (1987)
bSPI-DE4	Preparation DE-4 from bull seminal plasma	Inhibin-like	Sharma (1987)
LH-RBI	Luteinizing hormone receptor binding inhibitor, prepared from ovine corpora lutea	Inhibits binding of LH to its target cell receptors	Kumar, N., Dhir, R. N., Kumari, G. L. and Duraiswami, S. (unpublished results)
FSH-BI	Follicle stimulating hormone binding inhibitor, prepared from ovine follicular fluid	Inhibits binding of FSH to its target cell receptors	Dhir, R. N., Narender Kumar, Dhingra, S. R., Kumari, G. L. and Duraiswami, S. (unpublished results)

Statistical analysis:

The following statistics were performed: one way analysis of variance (ANOVA), standard correlation and regression analyses; differences among individual treatment means, after significant ANOVA main effects, were compared by the Student's Newman–Keul's (SNK) post hoc test (Sokal and Rohlf, 1969). A $P < 0{\cdot}05$ was considered statistically significant. The level of significance from the control is denoted by asterisks ($^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$).

with a log dose interval of 2, with 4 mice per dose in the case of the coupled assay and 3 wells per dose in the cell culture assay. The following criteria were applied for statistical validity: (i) index of significance of slope (g_c); (ii) variation of slope (G_c^2) and its F value (F_v); (iii) significance (t) of the differences of mean response and (iv) the index of precision (λ). Following Borth (1960) the level of statistical significance was set at 5%. Under these conditions, the combined slope for the assay is considered significant if the computed value for λ is <1. Variation in the slope and differences of mean responses are considered negligible if the calculated values are smaller than the table values for $P=0{\cdot}05$. An index of precision of 0·2 is generally considered acceptable for a bioassay. Since parallelism in the slopes of the log dose-responses were observed, the data was used for calculating relative potencies.

Results

Coupled assay

Inhibin/inhibin-like preparations (oTI, oFFI, bSPI-DE2 and bSPI-DE4) significantly inhibited uterine weight gain ($P<0{\cdot}001$, F-test) in a dose-dependent manner (figure 1) in the hCG-treated animals. The data exhibited highly significant linear regression ($P<0{\cdot}001$). The dosages required for 50% suppression of uterine weight as read off from the log dose-response curves (figure 2), are as follows: oTI = 7 μg; oFFI = 6·2 μg; bSPI − DE2 = 21 μg; bSPI − DE4 = 31 μg. Thus oFFI is slightly more active than oTI and the bSPI preparations. When these preparations were checked for their effect in hMG-treated immature mice, no significant suppression of uterine weight was observed even at as high a dose as 200 μg ($P>0{\cdot}75$, F-test, figure 3).

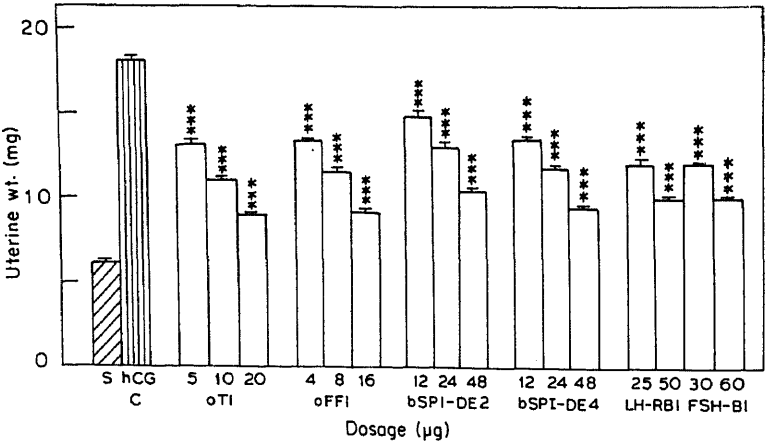

Figure 1. Effect of different preparations on hCG-induced uterine weight gain in the

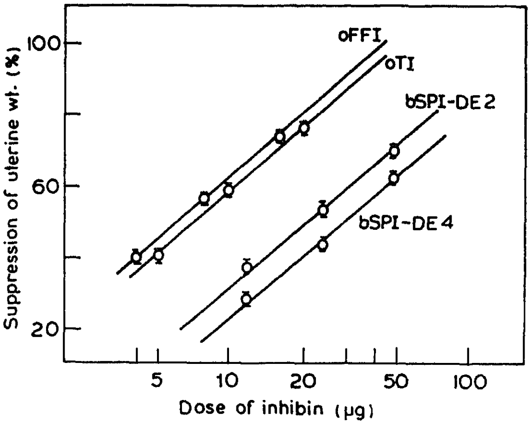

Figure 2. Log dose-response curves for the inhibin activity of different preparations tested at 3 dose levels in the hCG assay. The regression lines are based on statistical analysis as detailed in the text. The individual points however, represent percentage suppression of hCG-induced uterine weight increase (mean ± SEM, n = 4) for specified doses of the given preparation calculated from actual assay data. Percentage suppression was calculated by the following formula:

$$\% \text{ suppression} = \frac{(\text{Uterine weight of hCG control}) - (\text{Uterine weight of inhibin group})}{(\text{Uterine weight of hCG control}) - (\text{Uterine weight of saline control})} \times 100.$$

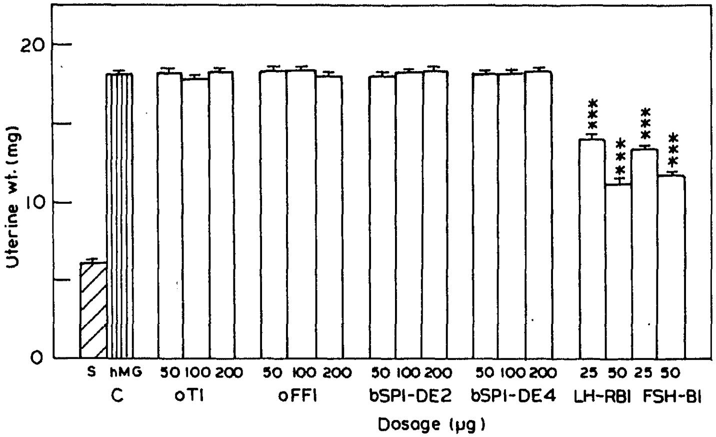

Figure 3. Effect of different preparations on hMG-induced uterine weight gain in the coupled assay. Bar chart represents mean ± SEM, n = 4. The inhibin-like preparations did not inhibit uterine response in the hMG-treated animals ($P > 0{\cdot}75$, F-test). On the other hand, preparations with LH-RBI/FSH-BI activity suppressed in a dose-dependent and statistically significant ($P < 0{\cdot}001$) manner the hMG-induced uterine weight gain.

In contrast, the receptor binding inhibitors (LH-RBI and FSH-BI) suppressed uterine weight gain in a dose-dependent manner not only in the hCG-treated but also in the hMG-treated animals ($P < 0{\cdot}001$, F-test, figures 1 and 3), when tested in the same dose ranges.

Pituitary cell culture assay

All inhibin-like preparations (oTI, oFFI, bSPI-DE2 and bSPI-DE4) also suppressed GnRH-stimulated FSH secretion into the medium significantly ($P<0{\cdot}001$, F-test, figure 4). FSH levels showed a highly significant linear regression with the dose ($P<0{\cdot}01$), deviation from linear regression being non significant ($P>0{\cdot}75$). On the other hand, receptor binding inhibitors (LH-RBI/FSH-BI) did not suppress GnRH-stimulated FSH secretion into the medium significantly ($P>0{\cdot}75$, F-test, figure 4). While the inhibin-like preparations sometimes suppressed GnRH-stimulated release of luteinizing hormone (LH) into the medium, no statistically significant dose-dependent effect could be observed ($P>0{\cdot}75$, F-test, figure 5).

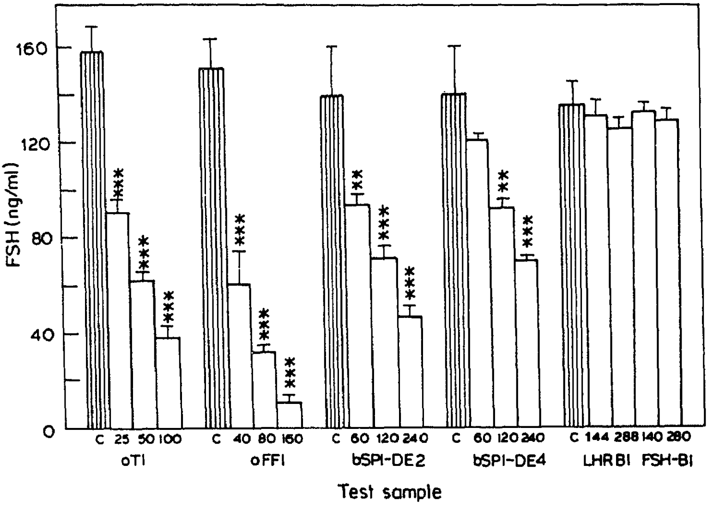

Figure 4. FSH levels in media of pituitary cells cultured for 6 days followed by 6 h of GnRH-stimulation. The bar chart represents mean ± SEM, n = 3. All inhibin-like preparations suppressed FSH in the medium significantly ($P<0{\cdot}001$, F-test) in a dose-dependent manner. On the other hand preparations with LH-RBI/FSH-BI activity did not suppress FSH in the medium ($P>0{\cdot}75$).

Figure 6 presents the log dose-response curves for the 4 inhibin preparations based on which the amount of protein required for 50% suppression by oFFI, oTI, bSPI-DE2 and bSPI-DE4 are found to be 28, 34, 120 and 190 ng respectively.

The possibility that a given test material might permanently damage the cells and thus reduce the secretion of FSH, was examined by replacing the medium containing test substances at the end of assay with fresh medium and assessing subsequent FSH output. The data presented in figure 7 are typical and indicate that the cells do recover their ability to secrete FSH into the medium after exposure to

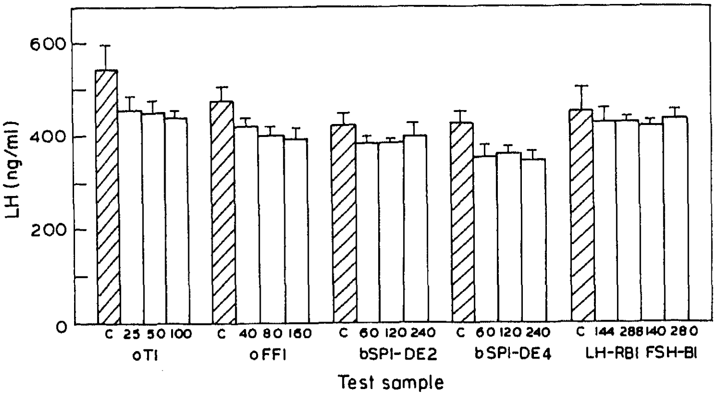

Figure 5. LH levels in the media of pituitary cells cultured for 6 days followed by 6 h of GnRH-stimulation. The bar chart represents mean ± SEM, n = 3. In no case was a statistically significant suppression observed ($P > 0{\cdot}75$).

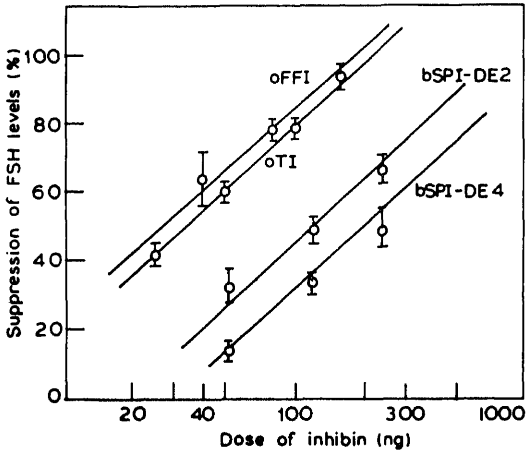

Figure 6. Log dose-response curves for ILA of different preparations tested at 3 dose levels in the pituitary cell culture assay. The regression lines are based on statistical analysis as detailed in the text. The individual points however, represent percentage suppression of GnRH-stimulated FSH levels in the medium (mean ± SEM, n = 3) for specified doses of the given preparation calculated from actual assay data. The percentage suppression was calculated as follows:

$$\%\ \text{suppression} = \frac{(\text{FSH level of control}) - (\text{FSH level of inhibin group})}{(\text{FSH level of control})} \times 100.$$

tical evaluation

data of the hCG-based and the *in vitro* pituitary cell culture assay was

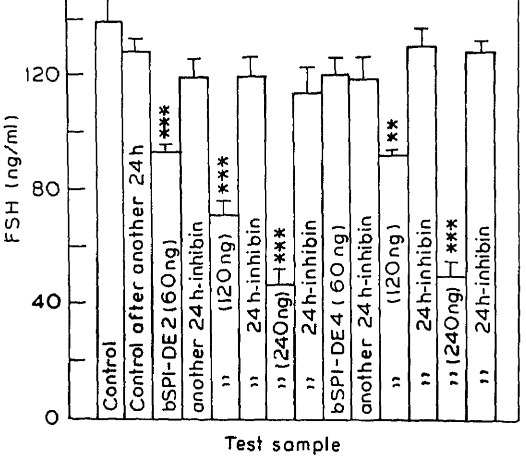

Figure 7. Demonstration of rebound FSH secretion by pituitary cells in culture following withdrawal of inhibin treatment. Controls, for this purpose, were the original controls (cells exposed to the 6-day culture + 6 h. GnRH exposure schedule), but which were then returned to the normal medium without additions and incubated for another 24 h. The bars labelled 'another 24 h-inhibin' represent those wells incubated for the prescribed 6 h in the presence of GnRH and inhibin, but returned for a final 24 h to normal medium not containing any other additions. Note that in all cases the cells have fully recovered their ability to secrete the 'control' levels of FSH.

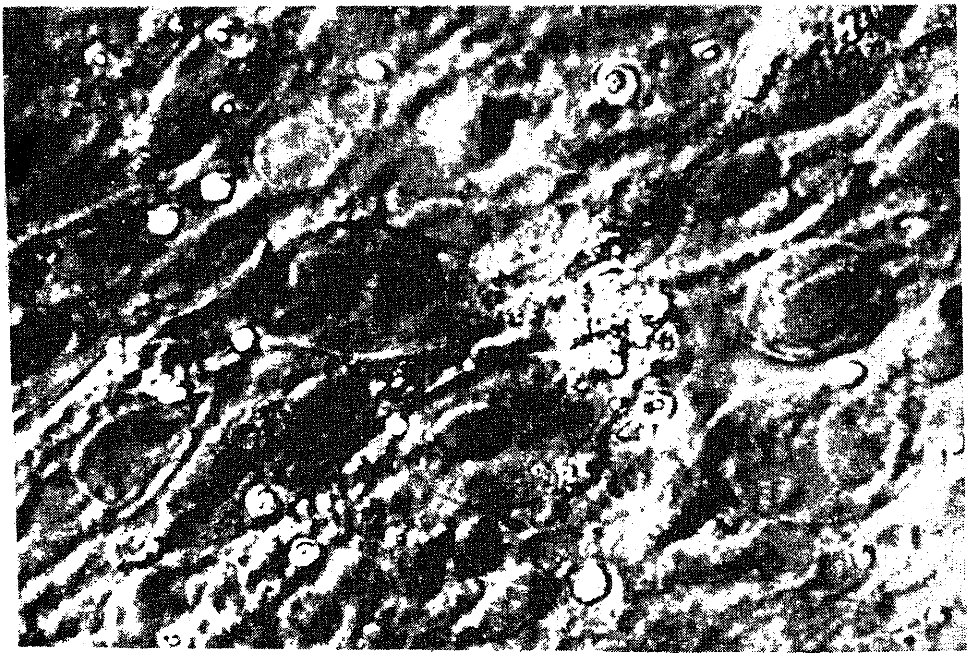

Figure 8. Phase contrast photomicrograph of pituitary cells at the end of 6 days of culture (× 500). Note the extension of cytoplasm of cells in an elongated form indicative of the viability of cells.

2. Statistical evaluation of the validity of the hCG-based and pituitary cell culture assay by 's system of computation.

Parameter										
n̄	J	I	b_c	S_c	S_c^2	V	g_c^*	G_c^2	$F_v^\dagger$	λ^{**}
hCG-based assay										
4	2	0·301	6·87	0·396	0·157	0·039	0·005	0·036	0·923	0·057
Pituitary cell culture assay										
3	2	0·301	76·83	8·91	79·3	26·4	0·026	27·13	1·03	0·116

erage number of animals per dose level; J, ratio of contiguous doses; I, log J; b_c, combined slope; andard deviation of response; S_c^2, residual variance; V, variance of the mean effects; g_c, index of cance of slope; G_c^2, departure from parallelism; F_v, F value for departure from parallelism. ex of precision.

computed value of g_c is less than 1 and therefore the combined slope for the assay can be lered significant.

computed value of F_v is less than the table value of 3·01. Therefore departure from parallelism is ible.

ne value of λ is less than 0·2 and therefore the index of precision of the assay is acceptable.

Table 3. Assessment of relative potencies of inhibin preparations.

	Preparation		
	oFFI	bSPI-DE2	bSPI-DE4
hCG based assay			
Relative potency (oTI as standard)	1·24	0·240	0·35
Fiducial limits	0·825–1·85	0·16–0·48	0·24–0·53
Pituitary cell culture assay			
Relative potency (oTI as standard)	1·46	0·340	0·17
Fiducial limits	1·13–2·1	0·26–0·46	0·15–0·22

had potencies of 0·2 to 0·4. These are in good agreement with the ratios ılated from log dose-response curves.

ne above results in addition to validating the coupled assay design, also signify gonadal inhibins are far more active than inhibin from extragonadal sources.

ussion

onale of the coupled assay

ıg intact as well as hypophysectomized immature female mice, Lamond and nens (1959) observed that the effect of hCG on uterine weight gain was virtually ished by hypophysectomy, suggesting the need for endogenously secreted ıdotropins. On the other hand hMG was able to elicit its response in both the ct as well as the hypophysectomized mouse. This is in line with the observations amond and Braden (1959) who demonstrated a diurnal variation in the uterine response of the immature mouse to hCG and pregnant mare serum gonadotropin but did not find it with hMG. Thus the response to hMG is not dependent on endogenous gonadotropins in the intact mouse. Using a more direct approach Ramasharma *et al.* (1979) demonstrated that hCG evokes an acute discharge of FSH which, by stimulating follicular maturation and synthesis of estrogen, causes an increment in uterine weight. Neutralization *in vivo* of the hCG induced FSH by a highly specific antiserum was found to abolish the hCG effect.

Based on these observations the assay of Ramasharma *et al.* (1979) was modified by the inclusion of a parallel assay in which hMG was injected instead of hCG so as to differentiate between inhibin-like preparations and those acting at the level of the gonads.

Validity of the coupled assay

From the foregoing it would be evident that if a test preparation suppresses uterine weight gain in hCG-treated animals but not in those treated with hMG, it is clearly inhibin in nature. On the other hand, if the hMG treated animals also respond by a suppression of uterine weight gain the preparation can be taken to act at the gonadal level. The data summarized in table 4, validates the argument on which the coupled assay design is based. Animals treated with oTI, oFFI, bSPI showed suppression of uterine weight gain only if they had been exposed to hCG. In contrast, animals treated with FSH-BI and LH-RBI preparations suppress uterine weight gain in both hCG and hMG-treated animals. These preparations have been shown already to be receptor binding inhibitors by appropriate radioreceptor assays (Kumari *et al.* 1984; Kumar, N., Dhir, R. N., Kumari, G. L. and Duraiswami, S., unpublished results).

The differential diagnosis effected by the coupled assay has been fully borne out by the results of the *in vitro* pituitary cell culture assay performed on the same preparations. Those preparations which suppressed hCG-induced but not hMG-induced uterine weight gain in the coupled assay were alone effective in suppressing GnRH-induced FSH secretion (see table 4). While inhibin-like preparations sometimes suppressed GnRH-stimulated release of LH into the medium, no statistically significant dose-dependent effect was observed. These results are in conformity with the observations of de Jong *et al.* (1979), Scott *et al.* (1982), Baker *et al.* (1981), LeGac and deKretser (1982) and Robertson *et al.* (1986).

Table 4. Statistical significance of the response as calculated by *F*-test.

Test sample	Coupled assay hCG	Coupled assay hMG	Pituitary cell culture assay
oTI	***	ns	***
oFFI	***	ns	***
bSPI-DE2	***	ns	***
bSPI-DE4	***	ns	***
LH-RBI	***	***	ns
FSH-BI	***	***	ns

***$P < 0.001$; ns, nonsignificant.

tistical evaluation

:allel dose-dependent responses have been obtained in the hCG-treated animals l in the pituitary cell culture assay. The statistical data shows that the linearity of dose response curve, the lack of deviation from parallelism of the dose response ves and the precision are within the acceptable range (table 2). The index of cision (λ) was found to be 0·057 for the coupled assay and 0·116 for the pituitary culture assay. The good agreement between the potencies of the different parations by the two assays permits the conclusion that the physiological basis he two assays must be the same, namely action of the active principle at the level pituitary to suppress FSH secretion.

formance and practicability

ibin bioassays based on the reversed Steelman–Pohley design have come in for icism on grounds of lack of specificity and reproducibility (Baker *et al.*, 1981; ady *et al.*, 1982). The coupled assay design has been shown to be specific as it is e to discriminate between true ILA and other interfering activities.

Based on the experience in this laboratory, several features were found to be portant for the reproducibility of the assay. Proper care in the maintenance of the use colony and control over the litter size and weight were crucial to roducibility. In addition reference to figure 9 would enable an appreciation of importance in this context of the temporal span of the assay. A preparation that wed highly significant dose-dependent suppression of uterine weight gain in the h assay design turned out to have no significant effect when assessed by the 3 injection schedule as used by Setchell and Sirinathsinghji (1972). Thus by this single modification a major source of variance could be avoided.

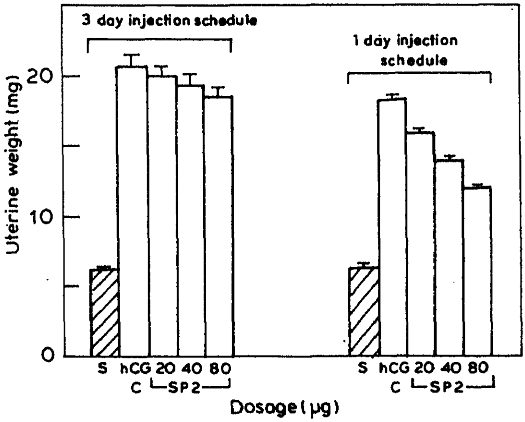

Figure 9. A comparison of the 4 day reversed Steelman–Pohley assay design for inhibin activity with the 24 h design used in this laboratory. Note that the preparation which gives a highly significant dose-dependent response in the 24 h schedule turns out to be marginally effective in the 4 day schedule.

The important shortcoming, namely low sensitivity, is none too serious in a

Therefore for the purpose for which it is designed it would appear to be eminently suitable, even though it is not anticipated that the coupled assay will replace the classical pituitary cell culture assay when plasma samples have to be assayed for their inhibin activity.

Acknowledgements

This work was done as part of the project: 'Studies on the physiology and biochemistry of inhibin and inhibin-like factors', supported by the Department of Science and Technology, New Delhi. SRD is a fellow of the Indian Council of Medical Research, New Delhi. The generous donation of the rat RIA kits for FSH and LH by the NIAMDD under the Indo-US sub-commission agreement is gratefully acknowledged.

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lative ability of ovine follicle stimulating hormone and its β-subunit generate antibodies having bioneutralization potential in nonhuman mates

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Abstract. The relative ability of ovine follicle stimulating hormone and its β-subunit, two potential candidates for male contraceptive vaccine, to generate antibodies in monkeys capable of bioneutralizing follicle stimulating hormone was assessed using *in vitro* model systems. Antiserum against native ovine follicle stimulating hormone was found to be highly specific to the intact form with no cross-reactivity with either of the two subunits while the antiserum against β-subunit of follicle stimulating hormone could bind to the β-subunit in its free form as well as when it is combined with α-subunit to form the intact hormone. Both antisera could block the binding of the hormone to the receptor if the hormone was preincubated with the antibody. However, the follicle stimulating hormone β-antisera could only inhibit the binding of the hormone partially (33% inhibition) if the antibody and receptor were mixed prior to the addition of the hormone, while antisera to the native follicle stimulating hormone could block the binding completely (100% inhibition) in the same experiment. Similarly antisera to the native follicle stimulating hormone was significantly effective in blocking (100%) response to follicle stimulating hormone but not the β-subunit antisera (0%) as checked using an *in vitro* granulosa cell system. Thus the probability of obtaining antibodies of greater bioneutralization potential is much higher if intact hormone is used as an antigen rather than its β-subunit as a vaccine.

Keywords. Gonadotropins; hormone action; receptor.

oduction

nunoneutralization of endogenous follicle stimulating hormone (FSH) (using er passive or active immunization approach) has been used in this laboratory in past as a means to gain insight into the mechanism of regulation of ovarian and icular function by FSH both in the rat and in the primate (Moudgal, 1981; ela Rani and Moudgal, 1983; Moudgal and Dighe, 1985). Based on these studies proposed developing ovine FSH (oFSH) as a contraceptive vaccine for use by human male (Moudgal *et al.*, 1986). Chronic neutralisation of circulating FSH ıdult male monkeys by specific antibodies has been shown to result in testicular function leading to infertility (Murty *et al.*, 1979). However, it was not clear from

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ority of the work reported here was carried out during the tenure of Visiting Scientist fellowship rded by the MRC Canada to the first author.

whom all correspondence should be addressed.

reviations used: FSH, Follicle stimulating hormone; oFSH, ovine FSH; a/s, antisera; PRP, iculate receptor preparation; PMSG, pregnant mare serum gonadotropin; RIA, radioimmunoassay; ;, human chorionic gonadotropin; LH, luteinizing hormone.

concentration and/or also by acting as an effective competitor of the receptor for binding FSH. Development of effective FSH vaccine is perhaps dependent upon the antigen under test producing antibodies capable of blocking FSH action at both the loci described above. The two candidate vaccines considered were intact FSH and its β-subunit. Since an antibody capable of blocking hormone-receptor interaction *in vitro* can be assumed to act as a good sequestering agent *in vivo* also, it was considered desirable to develop *in vitro* model systems wherein the ability of antibodies raised to different antigen to block hormone action can be studied. In the present study we have compared receptor binding inhibitory activity of an antiserum raised against native oFSH to that of an antiserum raised against oFSH β-subunit.

Materials and methods

Antisera

The antisera (a/s) used in these studies were raised in monkeys against highly purified oFSH (Sheela Rani and Moudgal, 1977) and its β-subunit (Sairam, 1979; Raj *et al.*, 1980). Information on the source and characterization of these a/s is provided.

Receptor preparation

The first part of the study was carried out using a particulate receptor preparation (PRP) prepared from pig granulosa cells (Sebok *et al.*, 1987). A pool of cells obtained during aspiration of pig follicular fluid was collected by centrifugation at 15,000 *g* for 30 min at 4°C, washed with 25 mM Tris HCl, pH 7·4, and homogenized in the same buffer using an all glass homogenizer. The suspension was finally lyophilized and stored at 4°C until used. Lyophilized preparations of the receptor have been kept stored for more than 5 years without loss of either [^{125}I]FSH or [^{125}I]hCG binding activity. Whenever necessary, suspension of this powder (20 mg/ml representing approximately 2 mg protein equivalent/ml) was prepared in 25 mM Tris-HCl, pH 7·4 containing 10 mM $MgCl_2$ and 0·1% bovine serum albumin (designated as RRA buffer).

Intact granulosa cells isolated from ovarian follicle of 23-day old female rats (Charles River, Canada) primed 48 h earlier with 15 IU/rat pregnant mare serum gonadotropin (PMSG) were also used as a source of the receptor. Following isolation, the cells were washed twice with Dulbecco's modified Eagle's medium and resuspended in a large volume of medium to provide approximately 250,000 cells per 0·1 ml aliquot. The cells were incubated with [^{125}I]oFSH in a total volume of 0·5 ml for different periods at 37°C in a Dubnoff metabolic incubator.

General methodology

Iodination of oFSH and subunits using carrier free [^{125}I]NaI was carried out cording to the procedure of Sairam (1978) and the specific activity of the eparation ranged from 50–80 μCi/μg. Each incubation was performed in plicate. The bound hormone was separated by centrifugation and the dioactivity in the pellet was determined using an autogamma spectrometer. aximum binding ability of each [^{125}I]oFSH preparation was determined by cubating the tracer with large excess of PRP and was found to vary between -25% of the input. Whenever needed, the antigen-antibody complex was precipi-ed using the appropriate second antibody.

Progesterone in the granulosa cell incubation mixture was assayed using a dioimmunoassay (RIA) system standardized and routinely used in the laboratory ato *et al.*, 1983).

All a/s dilution indicated here are the initial dilutions, 100 μl of each dilution tiserum being used per tube and the total volume being 500 μl.

sults

aracteristics of the a/s used

the 3 a/s raised against intact oFSH initially screened for their affinity, specificity d capacity, one antiserum was chosen for comparison with antiserum against the subunit of FSH. Antisera I (raised in a monkey against intact oFSH–NRM 603) owed high affinity (as determined by Scatchard analysis of binding data) for SH ($K_m - 1{\cdot}1 \times 10^{10}$ M$^-$) with a binding capacity of 130 μg oFSH/ml of serum. tisera II (a pool obtained from monkeys immunized against FSH β-subunit-GM 4) had relatively lower binding affinity for intact oFSH ($K_m - 0{\cdot}3 \times 10^9$ M$^-$), t the binding capacity (165 μg oFSH/ml) of this antiserum was almost similar to t shown by a/s I.

The cross reactivity of these two a/s with the subunits of oFSH was determined incubating 0·1 ml of different dilutions of a/s with [^{125}I]labelled α- and β-bunits of oFSH. Both the a/s showed insignificant binding to the α-subunit and en this could be observed only at the lowest dilution (1 : 100 dilution). Binding of ^{5}I]FSH β-subunit to a/s II was high, 50% binding being observed at 1 : 7000 ution. Relatively, 50% binding to intact [^{125}I]FSH was observed at a dilution of 2000. This is to be expected as number of binding sites in the free β-subunit can masked once the β-subunit comes in association with the α to make native FSH. tisera I was found to be more specific for native FSH and could bind labelled H β-subunit only at very low dilutions (50% binding of [^{125}I]oFSH β being served at a dilution of 1 : 300). These results showed that a/s I was mostly specific intact oFSH while a/s II could bind to intact oFSH through its β-subunit.

ect of antibodies on oFSH-receptor interaction using a PRP

eformed antibody [^{125}I]oFSH complex was unable to bind to FSH receptors ble 1). This was demonstrated by preincubating both the a/s (100 μl of 1 : 2000 ution of each a/s) with [^{125}I]oFSH for 2 h at 37°C prior to addition of receptor paration (2 mg PRP/tube) and continuing the incubation for additional 2 h at °C. This experiment essentially showed that either of the a/s at relatively the same

Table 1. Inability of [^{125}I]oFSH–Ab preformed complex to bind FSH receptors.

Incubated with a/s	cpm bound to receptor mean ± SEM	Inhibition (%)
Nonspecific binding	2126 ± 85	—
Specific binding (No a/s)	7944 ± 53	—
Antisera I (to intact oFSH)	2001 ± 34	100
Antisera II (to oFSH β)	2753 ± 268	89

The complex (hormone-antibody) was formed by incubating [^{125}I]oFSH with 100 μl of 1:2000 diluted respective antiserum for 60 min at 37°C. At this time 2 mg of a lyophylized porcine granulosa cell receptor preparation in 100 μl of RIA buffer was added and incubation continued for 120 min. Amount of [^{125}I]oFSH bound to the particulate receptor was determined.

concentration, despite having different binding affinities were capable of forming a firm complex with FSH, preventing its subsequent binding to FSH receptors.

Ability of the two a/s to compete with the receptor for binding [^{125}I]FSH was determined by premixing either of the a/s (100 μl of 1:2000 dilution) with the receptor preparation (2 mg PRP/tube) prior to addition of [^{125}I]oFSH. Receptor bound radioactivity was determined at the end of 2 h incubation at 37°C. While a/s I was able to inhibit 100% the binding [^{125}I]oFSH to its receptor, a/s II was able to inhibit, at the same dilution, the binding by only 33% (table 2).

The effect of varying the concentration of the antibody or of the particulate receptor preparation on the ability of [^{125}I]oFSH to bind the receptor was next determined. Using a constant receptor concentration (2 mg in a total volume of 500 μl) binding inhibition of 50% could be obtained with 100 μl of 1:5000 diluted a/s I (figure 1). Conversely the ability of the receptor protein to effectively compete with the antibody for labelled oFSH increased as the receptor concentration was enhanced, 8 mg of receptor protein (in a total volume of 500 μl) being required to overcome the 50% inhibition of binding obtained when 100 μl of 1:2000 diluted a/s I was used. Though the antibody was an effective competitor of the receptor for binding labelled oFSH, it was unable to dissociate the [^{125}I]oFSH prebound to the PRP even when tested at a high concentration (100 μl of 1:1000). This was demonstrated by adding the antiserum after different association times (from 30–240 min) of the hormone with the PRP (data not shown).

Table 2. Relative ability of different a/s to prevent [^{125}I]oFSH from binding to the receptor.

Identification of the a/s	cpm bound to the receptor* mean ± SD	Inhibition (%)
Nonspecific binding	3241 ± 50	—
Specific binding (No a/s)	13531 ± 513	—
Antisera I (to intact oFSH)	3229 ± 53	100
Antisera II (to oFSH β)	10084 ± 962	33

*Receptor preparation (2 mg/100 μl) and respective a/s (100 μl of 1:2000 dilution) were premixed before addition of [^{125}I]oFSH. Total volume of the reaction mixture was 500 μl and incubation time 2 h at 37°C. Results are mean of triplicate determinations. For the

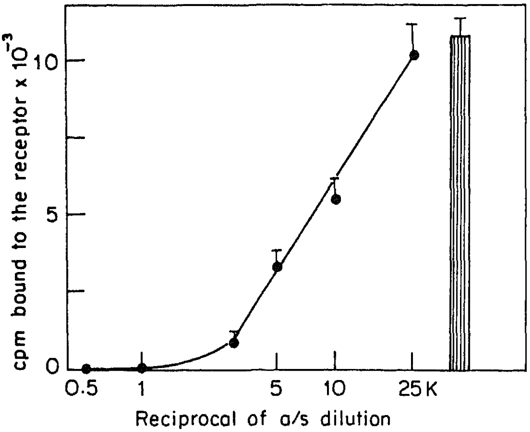

Figure 1. Effect of varying antibody concentration on the ability of [^{125}I]oFSH to bind to receptor.

[^{125}I]oFSH (approximately 100,000 cpm) was added to tubes containing 2 mg of PRP and 100 μl of different dilutions of a/s I and incubated for 2 h at 37°C. At the end of incubation the receptor bound radioactivity was determined. The nonspecific binding determined by incubating [^{125}I]oFSH with PRP in the presence of excess unlabelled oFSH was subtracted from the total binding observed. The bar indicates specific binding observed when [^{125}I]oFSH was incubated with PRP only. The values are mean ± SD of triplicate determinations.

eraction of [^{125}I]oFSH with its receptor in intact cells

anulosa cells isolated from ovaries of PMSG primed rats were preincubated with ^{5}I]oFSH for 30, 60 and 120 min at 37°C. After centrifugation and resuspension ı fresh medium, the cells were reincubated for 120 min in the presence or absence added a/s. It is evident from figure 2 that a significant portion of the bound mone (59% reduction in binding, $P < 0.001$) was released by a simple expedient incubation of the cells in a hormone free medium. Addition of antiserum I but a/s II to the incubation medium facilitated further discharge of the bound mone (82% reduction in the bound hormone). The experiment also showed that ociation time of the [^{125}I]oFSH with the granulosa cell had apparently no effect the ability of the cell to discharge the bound hormone. It was also observed that ibody could dissociate receptor-bound hormone in the intact cells while it was ıble to do so with particulate receptor (table 3).

ility of FSH a/s to disrupt FSH stimulated steroidogenesis in granulosa cells

ubation of rat granulosa cells with 50 ng FSH in the presence or absence of 10 μl normal monkey serum resulted in stimulation of production of progesterone. tiserum I was capable of inhibiting FSH response almost totally (98–100%) spective of whether it was preincubated with FSH or added subsequent to ing cells and FSH in the incubation medium (figure 3). Antiserum II, in ıtrast, was only partially effective in blocking FSH response (48–55% reduction progesterone produced, $P < 0.01$) and preincubation of FSH with the FSH β-ibody did not enhance the inhibition (figure 3).

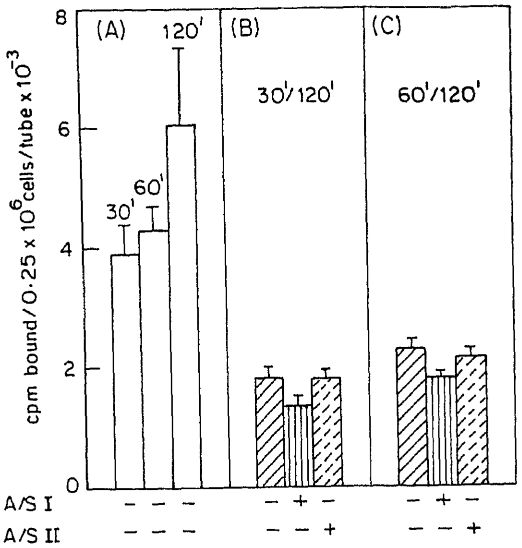

Figure 2. Ability of a/s to oFSH and its β-subunit to dissociate [^{125}I]oFSH boun
different periods to the rat granulosa cells.

Granulosa cells were incubated at 37°C for 30 and 60 min with [^{125}I]oFSH
incubation). The cells were centrifuged and binding was determined (panel A). The
were resuspended in fresh medium (0·5 ml) containing 10 μl of undiluted a/s I or II
reincubated for 120 min at 37°C (2nd incubation). At the end of incubation radioac
bound to the cells was determined. Panels B and C show the results when th
incubation was for 30 and 60 min respectively.

Table 3. Ability of a/s to discharge [^{125}I]oFSH bound to the receptor.

Receptor preparation	cpm bound to receptor	
	No a/s	With a/s I*
Particulate	4990 ± 455	5321 ± 220
Granulosa cells	4338 ± 417	1371 ± 120**

The labelled hormone was preincubated with the receptor preparation/cells for 1 h at 37°C prior to addition of a/s and continuing incubation for additional 2 h. The receptor/cell bound radioactivity was determined. The results are mean ± SD of triplicate determinations.

*Incubated with 10 μl of undiluted a/s.

**Significantly different from controls ($P < 0·001$).

The necessity of having FSH in the medium throughout the period of incuba
and ability of the a/s to stop the continued responsiveness of the cell to the bo
FSH was next tested. The results (figure 4) show that when granulosa
preincubated with FSH for 2 h were centrifuged and the medium replaced
fresh medium and incubation continued for additional 2 h, a significant reductio
progesterone production over the appropriate controls (52–55%, $P < 0·01$)
observed. Addition of a/s I to the medium during second incubation was effecti

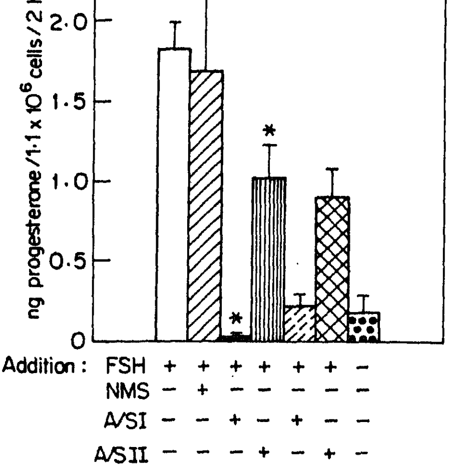

Figure 3. Ability of FSH a/s added at the beginning of the incubation on response of the granulosa cells to FSH.

Granulosa cells were incubated with either 10 μl NMS or a/s I or II in the presence of oFSH (50 ng) for 2 h at 37°C and progesterone secreted into the medium was estimated by RIA.

*FSH in these cases was preincubated with a/s for 30 min prior to addition of cells.

her inhibiting progesterone production (from 52–78%). The a/s II, however, was ce again ineffective in blocking steroidogenesis stimulated by the bound hormone ure 4).

cussion

primary objective of the current study was to understand whether antibodies able of sequestering FSH circulating *in vivo* are also able to inhibit FSH ponse *in vitro* and if so the mechanism by which this is achieved. All studies ried out hitherto on bioneutralization of endogenous FSH have used either well racterized polyclonal antibodies or in active immunization studies, a racterized antigen. The chances of obtaining bioneutralization *in vivo* is perhaps ater if more than one hormonal epitope is bound by its specific antibody. These erminants need not necessarily be those that interact with the receptor, as steric drances due to Ag–Ab complex at more than one locus could prevent the eptor recognition domain of the hormone from associating with the receptor.

The results of the present study have clearly shown that polyclonal bivalent ibodies do make excellent competitors of the receptor for binding to the mone. Of the 4 a/s initially screened two were chosen for detailed study as they ibited specific but different characteristics. It should be noted that both a/s I and ave been demonstrated in earlier studies to neutralize the bioactivity of FSH *in* (Murty *et al.*, 1979; Moudgal *et al.*, 1986, 1988; Raj *et al.*, 1980). Antiserum I by ue of its negligible binding to the free α- and β-subunits compared to its binding intact oFSH could be classified as conformation specific. Antiserum II, in

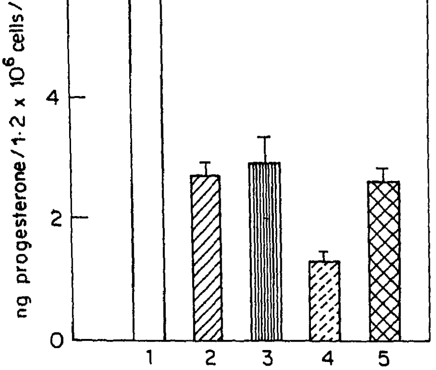

Figure 4. Ability of FSH a/s to disrupt ongoing steroidogenesis by granulosa cells under FSH stimulation.

The granulosa cells were preincubated with 50 ng FSH for 2 h. At the end of this incubation the cells were centrifuged, supernatant aspirated and the cells resuspended in medium (0·5 ml) containing either no addition, or with 10 μl each of NMS or a/s I or II and incubated for a further period of 2 h at 37°C. Progesterone secreted into the medium was estimated by RIA. Progesterone produced when FSH was incubated with cells for 2 h served as controls. The values are mean ± SD of triplicate determinations. (1), Controls (cells incubated with FSH for 2 h); (2), medium; (3), NMS; (4), a/s I; (5), a/s II.

contrast, by binding to the FSH β-subunit both in its free and combined state (intact FSH) showed that it is directed to specific sites in the β-subunit only. Though the binding capacity of a/s II was similar to a/s I, its binding affinity was relatively lower. While both the a/s used in this study were able to block completely the binding of labelled hormone to the particulate receptor preparation if the hormone was preincubated with the a/s, underscoring their *in vivo* sequestering property, their behaviour differed if the two binding entities (antibody and receptor) were mixed prior to the addition of [^{125}I]oFSH (table 2). It appears from this that the inhibition of binding was dependent more on their binding affinity than capacity. However, affinity may not be the only factor determining the inhibition potency as one of the other a/s raised to intact oFSH having nearly the same affinity ($\sim 0{\cdot}5 \times 10^9$ M$^-$) as a/s II, proved in competitive experiments to be as good an inhibitor of [^{125}I]FSH binding to the receptor as a/s I (data not shown). Poor inhibitory capacity can also then be a reflection of epitope specificity. Though the β-antiserum (a/s II) can be expected to have antibodies to a number of epitopes specific to its free state, it is possible that a majority of these epitopes are either masked or altered once the β-subunit recombines with the α to form native FSH. The epitopes recognized by a/s II in the whole hormone are perhaps those which are also directly recognized by the receptor resulting in only partial inhibition of the response. In the case of a/s I, however, binding of the antibody to conformationally specific site(s) should be preventing the association of the hormone with the receptor. It is interesting that the relative inability of the FSH β-a/s (a/s II) to block binding or response to FSH observed here is very similar to that exhibited by human chorionic gonadotropin (hCG) β-a/s in our earlier studies on hCG-receptor interaction (Dighe and Moudgal, 1981, 1983). We had postulated, based on those data, that initial recognition of hCG/luteinizing hormone (LH) by its receptor is occurring through sites specifically located in the β-subunit. The current study shows that this could be true for FSH also. It appears that for an antibody to be an effective inhibitor of hormone response it should bind site(s) involved in either direct interaction of the hormone with the receptor or to those located close to such sites. All the same in the present study the relative greater efficiency of the antiserum to intact FSH in competing with the PRP for binding of labelled hormone or blocking response of cells to FSH is perhaps due to antibodies binding the hormone at more than one loci/epitope making it difficult, because of steric consideration, for the hormone to associate with the receptor.

Another interesting observation that has emerged from the present study is that the interaction of FSH with its receptor varies depending upon the type of receptor used. While its binding to the particulate receptor preparation is tight even at early association times, its binding to cell-associated receptor is loose and a significant amount of the bound hormone dissociates upon incubating the cells in a hormone free medium. Unlike in the case of hCG where exposure of cells to the hormone for as short as 15 min is adequate to provide a full 2 h response (Dighe and Moudgal, 1983), continued association of FSH with the cellular receptor is essential for it to produce a sustained response. In this respect, oFSH appears to resemble PMSG and rat LH (Sheela Rani and Moudgal, 1985).

In conclusion, it can be stated that all polyclonal a/s capable of binding the hormone *per se* (radiolabelled or otherwise) need not necessarily be good competitors of hormone binding to the receptor. For complete bioneutralization *in vivo*, however, the antibodies in addition to being able to sequester the circulating hormone with high affinity should perhaps be also effective competitors of hormone binding to the receptor. Using the above criteria we believe intact oFSH rather than its β-subunit could turn out to be a better contraceptive vaccine. Studies carried out so far have shown that active immunization with intact oFSH results in antibodies which block binding of FSH to its receptors. These antibodies in turn cause infertility in the primate without having any effect on LH dependent functions underscoring the specificity of the vaccine (Moudgal *et al.*, 1986, 1988).

Acknowledgements

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Modulation of ovarian ornithine decarboxylase activity during follicular differentiation/maturation

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Abstract. The role of gonadotropins and estrogen on the regulation of ovarian ornithine decarboxylase was studied during follicular differentiation/maturation. In intact immature rats follicular differentiation/maturation was initiated with sequential administration of estrogen and follicle stimulating hormone. Ornithine decarboxylase activity in response to human chorionic gonadotropin was markedly enhanced (2-fold) in rats with preovulatory antral follicles when compared to rats with non-ovulatory follicles. This increase could be attributed to the alteration in the turnover of the enzyme. Following follicle maturation the half life of the human chorionic gonadotropin stimulated ornithine decarboxylase was increased from 18 to 62 min. This increase in half life was associated with differentition of follicles. In the estrogen treated group (which does not induce follicular differentiation), the half life of the enzyme remained unaltered. The regulation of ornithine decarboxylase through the formation of protein inhibitor antizyme induced by diamino hexane, was unaltered during follicular differentiation.

Keywords. Ovarian ornithine decarboxylase; follicular maturation; half life; antizyme.

Introduction

Mammalian ornithine decarboxylase (ODC), L-ornithine carboxylase (EC 4·1·1·17), catalyses the decarboxylation of L-ornithine to putrescine and carbon dioxide. It is the rate limiting step in the biosynthesis of the polyamines (Morris and Fillingame, 1974) ODC is known to have the shortest half life ($t\frac{1}{2}$) amongst all the mammalian enzymes (Morris and Fillingame, 1974).

Enhanced elaboration of polyamines associated with increased macromolecular biogenesis has been repeatedly observed. A large and rapid increase in ODC activity is a characteristic early event in several hormone stimulated target tissues including ovarian ODC stimulated by human chorionic gonadotropin (hCG) and luteinizing hormone (LH) (Kaye *et al.*, 1973). The regulation of ODC through the modulation of its $t\frac{1}{2}$ has been demonstrated in cultured mammalian cell lines (Hogan *et al.*, 1973). The present report demonstrates that the $t\frac{1}{2}$ of ovarian ODC changes dramatically in response to hCG during follicular differentiation/maturation.

Materials and methods

Pyridoxal-5′-phosphate (PLP), dithiothreitol (DTT), cycloheximide, diaminohexane and 17β-estradiol were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. [1-^{14}C]Ornithine monohydrochloride (52·8 mCi/mol), hyamine hydroxide were obtained from New England Nuclear, USA. [^{35}S]Methionine (800 mCi/mmol) was from Amersham, UK. Glass tubes (16 × 100 mm), and stoppers fitted with center wells were obtained from Kontes Glass Co., New Jersey, USA. All other chemicals used were of analytical grade.

Animals

Intact immature female rats procured from Holtzman, Chicago, USA were maintained on pelleted food and water *ad libitum.*

Initiation of follicular maturation and differentiation

Human follicle stimulating hormone (hFSH) (LER-1577), ovine LH (LER-B74) and hCG (Roussel corporation, 2950 IU/mg) were administered either subcutaneously or intravenously, in gel PBS (0·1% gelatin in 0·1 M phosphate buffer containing 0·14 M NaCl, pH 7).

All the experiments were carried out using intact immature female 24 day old Holtzman rats. Four groups each comprising of 4 rats were administered the following hormonal regime. Animals were injected 4 times at 12 h intervals with hFSH (1 μg) and 17β-estradiol (0·5, 0·5, 1 and 2 mg). This was the priming regime that resulted in a 2-fold increase in the ovarian weights associated with the development of large antral follicles (Richards *et al.*, 1976). Group 1 did not receive the priming regime but only a single dose of 10 IU hCG on day 26; group 2 following the priming regime, received 10 IU hCG on day 26. In addition groups 3 and 4 received four 12 h injections of estrogen (0·5, 0·5, 1, 2 mg) and hFSH (1 μg) respectively.

All the animals were sacrificed 4 h after the hCG injection and their ovaries removed for the measurement of ODC activity.

Preparation and assay of ODC

ODC activity was assayed according to Kaye *et al.* (1973). Briefly, the ovaries, granulosa cells or residual tissue were homogenized in isotonic sucrose (0·25 M), containing 25 mM Tris-HCl (pH 7·4), 5 mM DTT, 0·1 mM EDTA and 1 μM PLP. The homogenate was centrifuged for 30 min at 30,000 *g* in a Sorvall refrigerated centrifuge. The supernatant was used as an enzyme source. The standard enzyme assay contained 50 mM Tris-HCl (pH 7·4), 0·1 mM PLP, 5 mM DTT, 0·5 mM DL-ornithine containing 0·2 μCi of [1-^{14}C]ornithine·HCl and 0·1 ml of enzyme (50–500 μg protein) in a total volume of 500 μl. The enzyme was assayed at 37°C. The reaction was terminated by the injection of 1 ml of 10% trichloroacetic acid (TCA). The reaction was linear up to 2 h between a protein concentration of 10–2,000 μg. Protein was estimated by the method of Lowry *et al.* (1951).

nulosa cell expression

ovaries were trimmed off extra ovarian tissue and placed in cold PBS. nulosa cells were isolated in PBS by applying gentle pressure to the ovaries a blunt spatula. The rest of the ovary was designated residual tissue (Zeleznik *l.*, 1974). This tissue contained theca, interstitial cells and nonexpressed nulosa cells. The expressed granulosa cells and the residual tissue were washed e with PBS to remove follicular and interstitial fluid respectively.

tein synthesis and inhibition

synthesis of proteins was inhibited by the administration of cycloheximide ng/100 g body weight). The extent of protein inhibition was monitored by owing the incorporation of $[^3H]$leucine into the protein.

he average $t\frac{1}{2}$ of rapidly turning over protein was measured as follows. $[^{35}S]$ thionine (25 μCi/rat) and cyloheximide (3 mg/100 g body weight) were injected avenously at 3 and 4 h following the administration of hCG (10 IU/rat) and rats roups of 4 were sacrificed at specified time. The ovaries were homogenized in S and centrifuged at 25000 *g* for 30 min. The supernatant was subjected to 10% A precipitation, and the radioactivity in the TCA insoluble fraction was rmined.

ay of ODC-antizyme

presence of diaminohexane induced ODC inhibitor was monitored by the cedure of Heller *et al.* (1976). Briefly, at various time intervals after inistration of diaminohexane, the ovaries were removed homogenized with the dard homogenizing buffer (50 mM Tris, 1 mM DTT and 1 μM PLP) and rifuged at 25000 *g* for 30 min. The supernatant was dialyzed overnight against changes of buffer (50 mM Tris pH 7·4, 0·1 mM EDTA, 5 mM mercaptoethanol 0·1 μM PLP) at 4°C. The clarifed extract was added to a dialyzed standard paration of ODC (ovarian extract from 4 h hCG stimulated rats). The residual yme activity was determined. Appropriate controls were always included *i.e.*, yme activity of the extract alone.

ults

e course of hCG stimulated ovarian ODC during follicular differentiation/maturation

rian follicular differentiation and development was achieved by the hormonal tment regime detailed under materials and methods. The results are representa- of that obtained in two repetitive experiments. Figure 1A depicts the time rse changes of ODC activity following hCG administration in rats with non- latory (group 1) and preovulatory follicles (group 2). The ascending phase of the C stimulation was similar in both these groups. As early as 60 min after the

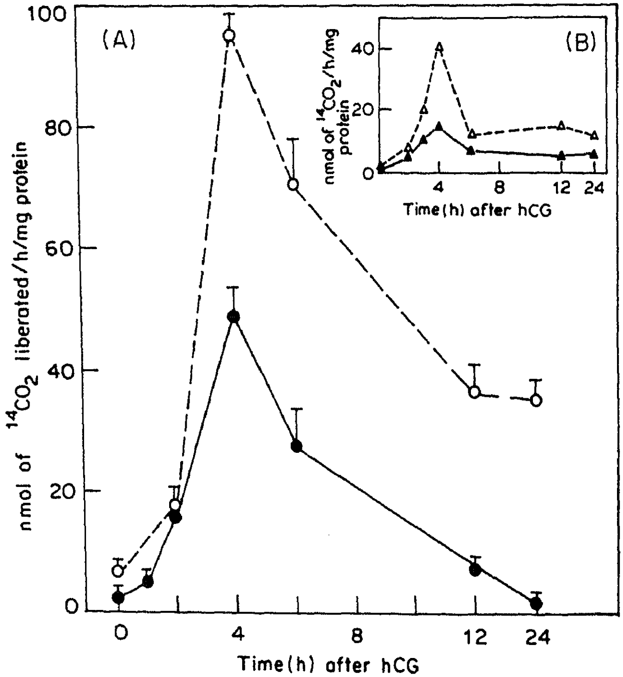

Figure 1. **A.** Stimulation of ovarian ODC by hCG. Intact immature rats (day 26) received a single intravenous injection of 10 IU of hCG. Ovarian ODC was estimated at various time intervals (group 1, ●). Intact immature rats (day 24) were treated with E_2 + FSH for 48 h to initiate follicular differentiation and maturation by the hormonal regime and on day 26 the rats received a single intravenous injection of 10 IU of hCG. Ovarian ODC was estimated at various time intervals (group 2, ○). **B.** ODC activity in dispersed granulosa cells (△) and residual tissues (▲) following hCG administration to rats (group 2). ODC activity was measured in granulosa cells pooled from 8 ovaries. Values are mean of duplicates.

was induced at 4 h and was 2-fold higher in group 2 (99 nmol/mg/h) than group 1 (49 nmol/mg/h). A more dramatic difference was observed in the descending phase. In unprimed animals the enzyme activity was indistinguishable from the basal endogenous activity 24 h after hormonal treatment while the activity was maintained at a high level 24 h after hCG injection in primed animals. A similar time course both in the ascending and descending phase of the curve was apparent both in the dispersed granulosa cells and the residual tissue (figure 1B).

$t\frac{1}{2}$ of ovarian ODC during follicular maturation

Figure 2A shows the rate of decay of ODC activity following inhibition of protein synthesis by cycloheximide. Cycloheximide (3 mg/100 g body weight) inhibits protein synthesis to more than 95% of the control level (1378 ± 120 cpm/mg to 68 ± 23 cpm/mg protein by 60 min, n = 5). The rate of decay of hCG induced ODC

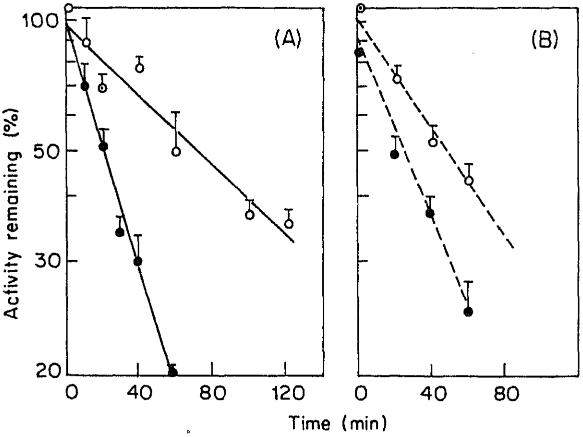

Figure 2. The $t\frac{1}{2}$ of ovarian ODC activity during follicular differentiation/maturation. The results are representative of that obtained in two repetitive experiments. 4 h following a single intravenous injection of hCG (10 IU), cycloheximide (3 mg/100 g body weight) was administered intraperitonealy and rats in groups of 4 were sacrificed and ovarian ODC activity measured at various time periods. **A.** Groups 1 (●) and 2 (○). **B.** Groups 3 (●) and 4 (○).

the hCG induced unprimed animals (group 1). However, in the primed treated with hCG (group 2), the $t\frac{1}{2}$ is dramatically increased to 62 min. 2B shows the rate of decay of hCG stimulated ODC in rats pretreated with strogen (group 3) or hFSH (group 4) and the $t\frac{1}{2}$ of hCG stimulated ODC and 40 min respectively.

sponse of diaminohexane induced inhibition of hCG stimulated ODC

3A represents the inhibition of hCG stimulated ODC by diaminohexane.

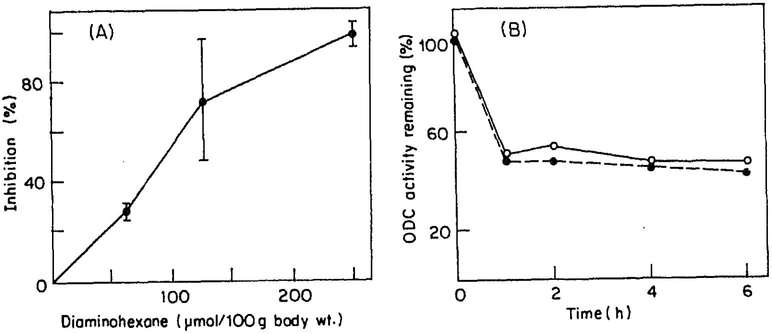

Figure 3. **A.** Dose response curve for the inhibition of hCG stimulated ovarian ODC activity by diaminohexane. Diaminohexance (μmol/100 g body weight) was administered intraperitoneally at 0 and 2 h to 26 day intact immature rats treated with hCG (10 IU). Ovarian ODC was estimated 4 h following hCG treatment (n = 5). **B.** Time course of appearance of ovarian ODC-antizyme in response to (125 μmol/100 g body weight) diaminohexane. The appearance of antizyme was estimated by measuring the extent of inhibition of activity. The antizyme was estimated at two different concentrations to establish linearity (n = 5). (○), Group 1; (●), group 2.

of 5 animals died following this treatment. Hence, a non lethal dose of 125 μmol/100 g body weight was selected for further studies. In rats treated with diaminohexane, the appearance of ODC antizyme was apparent. Figure 3B represents the time course of appearance of ODC antizyme. Antizyme formation was apparent by 1 h and the maximum was reached by 2 h and was maintained up to 6 h. A similar time course was seen both in groups 1 and 2.

Rate of decay of ODC by diaminohexane

Four h following hCG treatment 125 μmol of diaminohexane was administered and the residual enzyme activity was monitored (figure 4). The decay of ODC did not follow first order kinetics. The enzyme activity decreased after a lag period of 15 min. The latter half of the points could be fitted into a straight line. The slope of the lines were parallel both in unprimed (group 1) as well as in primed (group 2) animals following diaminohexane treatment, indicating that the rate of decay is unaltered by diaminohexane during follicular differentiation/maturation.

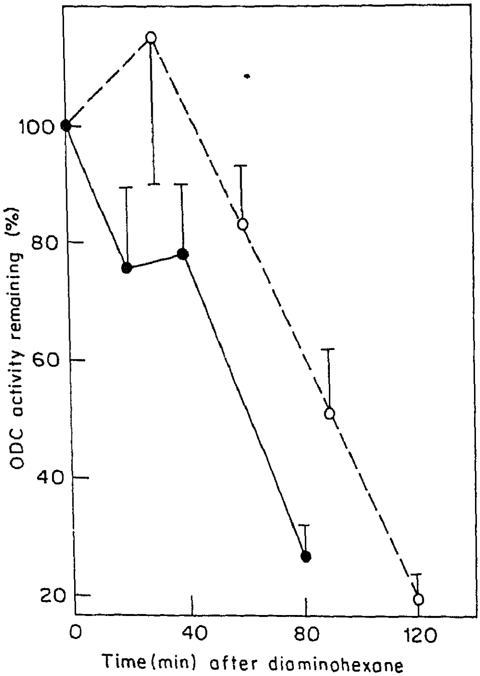

Figure 4. Rate of decay ODC by diaminohexane. 4 h after hCG administration to intact immature rats, diaminohexane (125 μmol/100 g body weight) was administered and the residual ODC activity was estimated. Values are mean ± SEM (n = 5). (●), Group 1; (○), group 2.

Average $t_{\frac{1}{2}}$ of rapidly turning over protein in hCG stimulated animals

In unprimed (group 1) and primed (group 2) animals, 3 h following hCG treatment,

]methionine (25 μCi/rat) was administered to label the protein. One h after this, heximide (3 mg/100 g body weight) was injected intraperitoneally to inhibit al protein synthesis and the average $t\frac{1}{2}$ of [^{35}S] labelled soluble proteins were ined. Figure 5 indicates a difference in the slope of the decay [^{35}S] labelled ins in group 2 as compared to group 1.

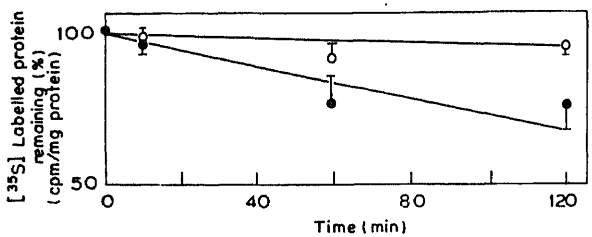

Figure 5. The average $t\frac{1}{2}$ of ovarian proteins, 3 h after hCG (intravenous, 10 IU) [^{35}S]methionine (25 μCi/rat) was administered (intraperitoneally) to label the proteins. 1 h following this, cycloheximide (3 mg/100 g body weight) was administered. Ovaries were removed, homogenized and centrifuged at 25,000 *g* for 30 min. The supernatant was subjected to 10% TCA precipitation. The radioactivity in the TCA insoluble fraction was determined cpm/mg protein was calculated. Values are mean ± SEM (n = 5). (●), Group 1 (○), group 2.

ussion

increase in ovarian ODC in response to LH/hCG has been known for some (Koboyashi *et al.*, 1971; Kaye *et al.*, 1973; Nureddin, 1977; Osterman and mond, 1977). ODC is known to have the shortest $t\frac{1}{2}$ amongst mammalian mes. The $t\frac{1}{2}$ of hCG induced ovarian ODC in intact immature rat is 18 min e *et al.*, 1973). The LH/hCG receptor content undergoes alteration during ular maturation from the preantral to antral follicles. Estradiol and FSH are arily responsible for these changes (Richards, 1979). Estrogen and FSH inistration to either intact or hypophysectomized immature rats results in the feration of granulosa cells, acquisition of LH receptors and the ability to ate in response to an ovulatory dose of hCG (Rao *et al.*, 1978). There is a lation between LH stimulated ODC activity and the content of LH receptor te and Ojeda, 1981). This model was utilised to study the hormonal regulation arian ODC during follicular differentiation/maturation.

the intact immature rats, LH/hCG stimulated ODC in a time and dose ndent manner, and when treated with estradiol for two days in the absence of , the basal enzyme activity was decreased. Estrogen induced granulosa cell feration was accompanied with the reduction in LH receptors (Rao *et al.*,). However, these animals respond to hCG/LH in stimulating ovarian ODC the enzyme, (iii) synthesis of different forms of the enzyme with altered stability *in vivo* and *in vitro* and (iv) changes in the general protein synthesis and degradation.

The enhanced ODC activity following follicular maturation could be accounted by the increase in hCG/LH receptor in the granulosa cells (White and Ojeda, 1981). Apart from the increase in the rate of synthesis, the regulation of the level of the enzyme by changes in $t\frac{1}{2}$ of the enzyme has been shown to occur in a number of cell lines (Poso *et al.*, 1978; Seely *et al.*, 1982). Preliminary studies indicated that the $t\frac{1}{2}$ of ODC in granulosa cells and residual tissue were similar and hence in the subsequent studies whole ovaries were used. Data in figure 2 clearly indicate that the $t\frac{1}{2}$ of hCG stimulated ODC is dramatically altered during follicular differentiation/maturation from 18 to 62 min. Estrogen alone which had no effect on follicular differentiation did not alter the $t\frac{1}{2}$ of ODC and FSH does increase the $t\frac{1}{2}$ to 40 min. In these experiments, the decrease in the catalytic activity was examined rather than the enzyme mass. There is evidence to show that a decrease in catalytic activity reflects the decay in enzyme mass implying thereby that the changes in the $t\frac{1}{2}$ could reflect enzyme mass (Canellakis and Theoharides, 1976; Holtta, 1975; Obenrader and Prouty, 1977; Seely *et al.*, 1982).

The enzyme is known to be regulated by a protein, 'antienzymol' induced by polyamines (Heller *et al.*, 1976). Thus, it was argued that the regulation by antizyme during follicular differentiation/maturation could also be altered. This possibility was explored. Among the 4 polyamines (putrescine, cadavarine, spermidine and diaminohexane) tested for their ability to induce ODC antizyme, the synthetic polyamine diaminohexane was effective in stimulating ODC antizyme. The time course of antizyme formation, the dose response in groups 1 and 2 were similar. Further the decrease in enzyme activity following the administration of diaminohexane was similar in both of these groups, thus indicating that the regulation of ODC by antizyme was not altered during follicular differentiation and maturation.

Recently, it has been shown that ODC of the human neuroblastoma cell line (Paju) had an unusual $t\frac{1}{2}$ (>8 h) and that this was due to the synthesis of enzyme with altered molecular weight (Poso *et al.*, 1985). It has also been suggested that there may exist ODC with differences in temperature stability. Data are not at hand to rule out these possibilities but the *in vitro* stability of the hCG stimulated ODC at 37°C was similar in both these groups (data not shown).

The fourth possibility that a change in the general protein synthesis/decay was altered during follicular differentiation/maturation was examined by estimating the average $t\frac{1}{2}$ of the newly synthesised proteins. The results suggest that follicular differentiation/maturation is associated with an increase in the average $t\frac{1}{2}$ ($P<0{\cdot}05$) of proteins and more dramatically in ODC. Although it has been demonstrated that the turn over of hCG induced ovarian ODC is altered during follicular differentiation/maturation, the significance of these changes is not very clear. A number of investigators have enumerated the general role of polyamines in growth and development (Morris and Fillingame, 1974), but whether ovarian ODC has a specific role in follicular maturation needs to be evaluated. The principal function of the ovary is steroidogenesis, and during follicular differentiation/maturation, a shift from estrogen to progesterone synthesis occurs. It is therefore tempting to speculate that ODC and/or polyamines may have some role in these key events.

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X-ray studies on crystalline complexes involving amino acids and peptides: Part XVIII. Crystal structure of a new form of L-arginine D-glutamate and a comparative study of amino acid crystal structures containing molecules of the same and mixed chirality

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Abstract. The new form of L-arginine D-glutamate is monoclinic, $P2_1$, with $a = 9{\cdot}941(1)$, $b = 4{\cdot}668(2)$, $c = 17{\cdot}307(1)$ Å, $\beta = 95{\cdot}27(1)^\circ$, and $Z = 2$. In terms of composition, the new form differs from the old form in that the former is a monohydrate whereas the latter is a trihydrate. The structure has been solved by the direct methods and refined to $R = 0{\cdot}085$ for 1012 observed reflections. The conformation of the arginine molecule is the same in both the forms whereas that of the glutamate ion is different. The change in the conformation of the glutamate ion is such that it facilitates extensive pseudosymmetry in the crystals. The molecules arrange themselves in double-layers stabilised by head-to-tail sequences involving main chains, in both the forms. However, considerable differences exist between the two forms in the interface, consisting of side chains and water molecules, between double-layers. A comparative study of the relationship between the crystal structures of L and DL amino acids on the one hand and that between the structures of LL and LD amino acid-amino acid complexes on the other, provides interesting insights into amino acid aggregation and the effect of chirality on it. The crystal structures of most hydrophobic amino acids are made up of double-layers and those of most hydrophilic amino acids contain single layers, irrespective of the chiralities of the amino acids involved. In most cases, the molecules tend to appropriately rearrange themselves to preserve the broad features of aggregation patterns when the chirality of half the molecules is reversed as in the structures of DL amino acids. The basic elements of aggregation in the LL and the LD complexes are similar to those found in the crystals of L and DL amino acids. However, the differences between the LL and the LD complexes in the distribution of these elements are more pronounced than those between the distributions in the structures of L and DL amino acids.

Keywords. Amino acid complexes; effect of chirality; amino acid aggregation; head-to-tail sequences of amino acids; chemical evolution.

Introduction

We have been pursuing a programme of x-ray studies on crystalline complexes involving amino acids and peptides in order to define, at the atomic resolution, the geometrical features of biologically important non-covalent interactions (Salunke and Vijayan, 1984; Suresh and Vijayan, 1985; Suresh *et al.*, 1986). This work has led, among other things, to the elucidation of specific interactions and characteristic interaction patterns involving functional groups found in proteins (Sudhakar *et al.*, 1980; Sudhakar and Vijayan, 1980; Salunke and Vijayan, 1981, 1983; Vijayan, 1983). It has also resulted in a new approach to prebiotic phenomena based on molecular interactions and aggregation (Vijayan, 1980; Vijayan and Suresh, 1985). The current focus of the programme is on complexes of amino acids of mixed chirality. In this context we have already analysed the crystal structures of 5 LD complexes, namely, L-arginine D-glutamate trihydrate (Suresh *et al.*, 1986), L-arginine D-aspartate monohydrate (Soman *et al.*, 1988) and L-ornithine D-aspartate monohydrate (Soman and Vijayan, 1988), and two DL-DL complexes, namely, DL-arginine DL-glutamate monohydrate (Soman *et al.*, 1989) and DL-arginine DL-aspartate (Soman *et al.*, 1989). The comparison of these structures with those of the corresponding LL complexes has provided valuable insights into the effect of chirality on amino acid aggregation and its possible relevance to chemical evolution. Here we report the crystal structure of a new form of a complex between L-arginine and D-glutamic acid, which contains only one water molecule per a pair of amino acid molecules instead of 3 in the complex the structure of which has already been analysed.

Having analysed several LL and LD amino acid complexes, it is of obvious interest to investigate if the relation between the two sets of complexes bears any resemblance to that between the structures of L amino acids and the corresponding DL amino acids. Indeed no systematic and comprehensive study of the relationship between the crystal structures of L and DL amino acids appears to have been carried out. In the second part of this paper, this relationship is explored, and the aggregation patterns in the LL and LD complexes are discussed with reference to it.

Materials and methods

X-ray analysis

The complex between L-arginine and D-glutamic acid was originally crystallised from an aqueous solution of the components in molar proportions by the diffusion of acetone into it (Suresh *et al.*, 1986). In addition to the normal crystals the structure of which was reported earlier, the crystallization dish contained a single crystal with a different morphology. Repeated attempts to reproduce more crystals of the latter type were of no avail. Therefore, the one available crystal was used for all subsequent X-ray work. This crystal was needle-like in shape and had dimensions 0·10 × 0·15 × 0·70 mm. It was found to belong to the monoclinic space group $P2_1$ with $a=9{\cdot}941(1)$, $b=4{\cdot}668(2)$, $c=17{\cdot}307(1)$ Å and $\beta=95{\cdot}27(1)^\circ$. The old form also belonged to the space group $P2_1$ with $a=9{\cdot}968$, $b=4{\cdot}652$, $c=19{\cdot}930$ Å and $\beta=101{\cdot}20^\circ$. Thus the difference in the cell dimensions is primarily in the length of the *c* axis. The intensity data from the new crystal were collected on a CAD-4 computer controlled diffractometer using nickel-filtered CuKα radiation up to a Bragg angle of 65° with $h=0$ to 11, $k=0$ to 5 and $l=-19$ to 19. The data were corrected for Lorentz and polarisation factors. Intensities of $0kl$ and $0k\bar{l}$ reflections yielded a merging R of 3%.

The structure solution turned out to be more difficult than anticipated. In retrospect, the extreme pseudosymmetry (see later) appears to have caused the difficulty. The solution obtained using the direct methods program MULTAN (Main *et al.*, 1980) contained what appeared to be peaks corresponding to two arginine molecules instead of an arginine molecule and a glutamate ion. It also contained 4 unexplained peaks. The final correct solution was achieved through several trial calculations, including a few for an averaged structure in space group $P2_1/a$, and examination of several difference Fourier maps. The structure thus ained was refined using the block-diagonal SFLS method. The non-hydrogen ms were refined anisotropically and the hydrogen atoms, fixed from a difference urier map using geometrical considerations, isotropically. Form factors for non-lrogen atoms were taken from Cromer and Waber (1965) and those for hydrogen ms from Stewart *et al.* (1965). Refinement was terminated at a R factor of 0·085 1012 observed reflections with $I > 2\sigma$ (I). The weighting scheme used had the n $1/(a+bF+cF^2)$ with $a=0{\cdot}164$, $b=0{\cdot}111$ and $c=0{\cdot}003$. The final difference urier map had no positive features $>0{\cdot}48$ eÅ^{-3}. Table 1 lists the final positional ameters and the equivalent isotropic temperature factors (Hamilton, 1959) of the 1-hydrogen atoms.

Table 1. Positional parameters (X10000) and equivalent isotropic temperature factors of non-hydrogen atoms in L-arginine D-glutamate monohydrate.

Atom	X	Y	Z	Equivalent B
N1	6300(8)	2979	4128(5)	2·0(2)
O1	4348(7)	−848(16)	4379(4)	2·5(2)
O2	2751(7)	2072(17)	3830(5)	2·9(2)
C1	3952(11)	1318(24)	3980(6)	2·3(3)
C2	5037(11)	2968(23)	3595(6)	2·2(3)
C3	5259(10)	1520(22)	2834(5)	1·6(2)
C4	6373(10)	2924(25)	2387(7)	2·5(3)
C5	6777(10)	889(29)	1755(6)	2·5(3)
N6	7816(10)	1931(23)	1324(5)	2·7(2)
C7	7691(9)	3750(29)	708(5)	2·0(3)
N8	6476(9)	4774(24)	469(5)	2·8(2)
N9	8735(10)	4370(28)	356(6)	3·3(3)
N11	1311(7)	−2942(19)	4102(4)	1·5(2)
O11	−662(7)	708(18)	4402(4)	2·3(2)
O12	−2252(7)	−2046(18)	3810(5)	3·0(2)
C11	−1060(9)	−1368(22)	3983(5)	1·1(2)
C12	39(9)	−3015(21)	3605(6)	1·6(2)
C13	229(10)	−1620(25)	2820(6)	2·3(3)
C14	1319(12)	−2640(28)	2362(3)	2·6(3)
C15	1509(10)	−1041(24)	1649(6)	1·9(2)
O16	583(9)	529(23)	1339(5)	3·7(2)
O17	2644(8)	−1217(20)	1368(5)	3·3(2)
W1	3767(9)	3728(20)	887(5)	3·7(2)

Estimated SD are given in parentheses.

sults and discussion

olecular and crystal structure of the complex

olecular dimensions: The bond lengths and angles in the zwitterionic positively arged arginine molecules and the zwitterionic negatively charged glutamate ions by and large normal. There are a few departures from standard values (Vijayan, 76) but they do not merit special comment in view of the presence of pseudo-nmetry in the crystals and the comparatively high SD. The torsion angles that define the conformation of the molecules (IUPAC-IUB Commission on Biochemical Nomenclature, 1970) have the following values:

L-Arg: $\psi' = -36{\cdot}8(12)$; $\psi'' = 148{\cdot}6(10)$; $\chi^1 = -58{\cdot}9(11)$
$\chi^2 = 165{\cdot}0(9)$; $\chi^3 = -178{\cdot}4(9)$; $\chi^4 = -82.6(14)$
$\chi^{51} = 1{\cdot}3(17)^\circ$
D-glu: $\psi' = 31{\cdot}2(11)$; $\psi'' = -153{\cdot}7(9)$; $\chi^1 = 53.8(12)$
$\chi^2 = -175.1(9)$; $\chi^{31} = -19{\cdot}2(15)^\circ$

The side chain of the arginine molecule assumes sterically the most favourable orientation (Bhat *et al.*, 1979) in which it is *trans* to the α-carboxylate group. The side chain has an extended conformation except for the guanidyl group which introduces a fold at the terminus of the side chain with χ^4 assuming a value close to -90°. The observed conformation is one of the 15 unique conformations of arginine identified so far in crystal structures (Ramachandran *et al.*, 1965; Suresh *et al.*, 1986; Prasad, G. S. and Vijayan, M., unpublished results) and is the same as that found in the original form of the LD complex between arginine and glutamic acid. The glutamate ion in the structure has sterically the most favourable conformation with an all *trans* side chain *trans* to the α-carboxylate group, a conformation observed in several crystal structures (Bhat and Vijayan, 1977; Sequeira *et al.*, 1972; Soman *et al.*, 1988), but not in the original form of the complex. Thus L-arginine has the same conformation in the two forms but the D-glutamate ion has different conformations in them. It is also interesting to note that ψ', χ^1 and χ^2 in the L-arginine molecule and the D-glutamate ion in the present structure have nearly the same magnitudes but opposite signs. The same is true of χ^3 in the arginine molecule and χ^{32} in the glutamate ion. This feature has interesting consequences in terms of pseudosymmetry.

Molecular aggregation, hydrogen bonding and pseudosymmetry: The crystal structure of the complex is illustrated in figure 1. The parameters of the hydrogen bonds that stabilise the structure are given in table 2. As in the case of the original complex, the amino acid molecules form double-layers parallel to the *ab* plane in the structure. Each layer in the double-layer is made up of L-arginine molecules and D-glutamate ions and is stabilised by two DL head-to-tail sequences (Suresh and Vijayan, 1983). The two layers in the double-layer are held together by zigzag head-to-tail sequences, one involving L-arginine molecules and the other involving D-glutamate ions. Thus, as in the original form, the core of each double-layer is made up exclusively of main chain atoms interconnected through head-to-tail sequences, and is flanked by side chains. In fact, the structure and the interactions in the core are identical in the two structures.

The original form of the complex contains 3 water molecules and the interactions between double-layers are entirely through water bridges which connect side chains. The new form, however, contains only one water molecule and the interactions between double-layers involve water bridges and side chain-side chain interactions. The interface between the double-layers and indeed side chain-side chain interactions in general, are substantially different in the two structures. In the present structure, the interface contains a straight N–H---O hydrogen bond between side chain guanidyl and carboxylate groups, and a water bridge involving side chains. The water molecule also links side chains of adjacent molecules in the

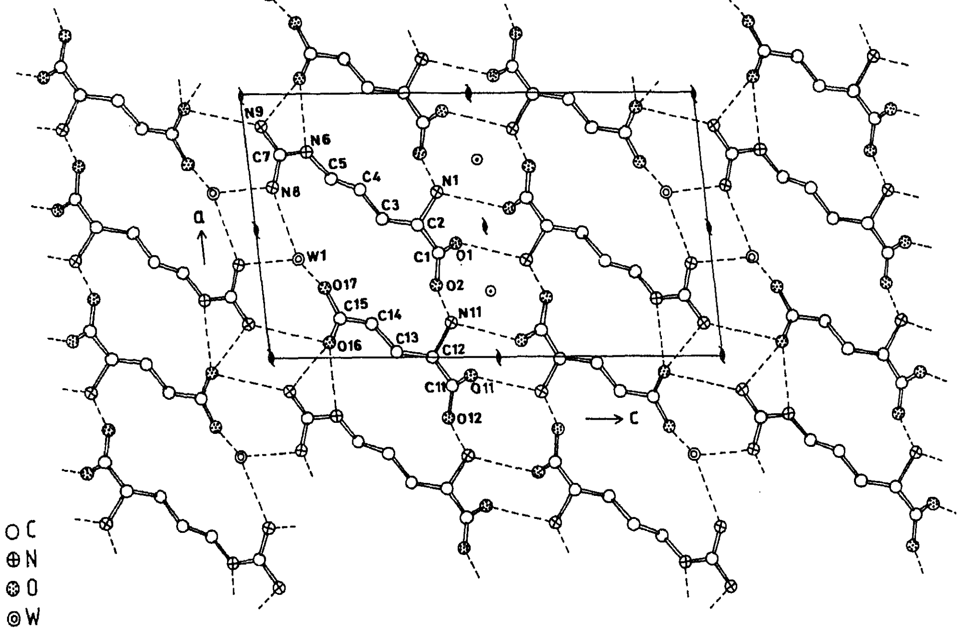

Figure 1. The crystal structure of L-arginine D-glutamate monohydrate as viewed along the *b* axis. Broken lines denote hydrogen bonds. In this and the subsequent figures, hydrogen bonds connecting molecules related by a translation along the direction of projection have been omitted for clarity. The dotted circles represent pseudo inversion centres.

Table 2. Hydrogen bond parameters in L-arginine D-glutamate monohydrate.

A–H . . . B	A . . . B(Å)	H–A . . . B(°)	Symmetry of atom B
N1–H1(N1) . . . 012	2·83(1)	25(8)	x+1, y, z
N1–H2(N1) . . . 012	2·81(1)	23(7)	x+1, y+1, z
N1–H3(N1) . . . 01	2·77(1)	2(10)	−x+1, y+1/2, −z+1
N6–H1(N6) . . . 016	2·83(1)	15(7)	x+1, y, z
N8–H1(N8) . . . W1	2·89(1)	12(7)	x, y, z
N8–H2(N8) . . . W1	2·98(1)	11(8)	−x+1, y+1/2, −z
N9–H1(N9) . . . 016	2·99(1)	33(8)	x+1, y, z
N9–H2(N9) . . . 016	3·12(1)	24(8)	−x+1, y+1/2, −z
N11–H1(N11) . . . 011	2·80(1)	9(6)	−x, y−1/2, −z+1
N11–H2(N11) . . . 02	2·79(1)	28(7)	x, y−1, z
N11–H3(N11) . . . 02	2·81(1)	14(7)	x, y, z
W1–H1(W1) . . . 017	2·73(1)	14(9)	x, y, z
W1–H2(W1) . . . 017	2·77(1)	6(10)	x, y+1, z

Estimated SD are given in parentheses.

same layer. Yet another link between molecules within a layer is a type D specific interaction (Salunke and Vijayan, 1981) between side chain guanidyl and carboxylate groups.

Many complexes containing amino acids of mixed chirality exhibit pseudo symmetry in their crystals (Suresh *et al.*, 1986; Soman *et al.*, 1988, 1989). For example, in the old form of the L-arginine D-glutamate complex, the main chain atoms in L-arginine and those in D-glutamate are related by a pseudo inversion centre midway between the two 2_1 screw axes in the middle of the double-layer. The pseudo inversion centre and the crystallographic 2_1 screw axis combine to give a pseudo *a* glide perpendicular to *b*. Thus the old form has a pseudosymmetry of $P2_1/a$ when only the main chain atoms are considered. The same pseudosymmetry not only exists but extends to a substantial part of the side chains also in the present structure. The change in the conformation of the glutamate ion is such that the side chain atoms C3, C4, C5 and N6 in the arginine molecule are pseudosymmetrically related to the side chain atoms C13, C14, C15 and O17 of the glutamate ion. Thus 18 out of the 23 non-hydrogen atoms in the structure obey pseudo $P2_1/a$ symmetry.

Comparison of amino acid structures containing molecules of the same and mixed chirality

The relationship between the aggregation patterns in LL amino acid-amino acid complexes and those in the corresponding LD complexes has been investigated in some detail through studies in our laboratory (Suresh *et al.*, 1986; Soman *et al.*, 1988; Soman and Vijayan, 1988). The differences between a LL complex and the corresponding LD complex result from the reversal in the chirality of half the molecules, all of the same type, in the aggregate. The differences between the crystal structure of an L amino acid and that of the corresponding DL amino acid also result from the reversal in the chirality of half the molecules in the aggregate, although in this case both the isomers are of the same type. The two situations are thus somewhat similar. Therefore, a careful comparative study of the aggregation patterns in the crystal structures of L and DL amino acids, and a re-examination of the patterns in the LL and LD complexes on the basis of the results of this study, would be of some value in exploring the effect of chirality on molecular aggregation.

L and DL amino acid crystal structures: Before going into the detailed analysis of aggregation patterns in amino acids and their complexes, a brief introduction to the nomenclature of head-to-tail sequences (Suresh and Vijayan, 1983), in which the α-amino and the α-carboxylate groups of adjacent molecules are brought into hydrogen-bonded proximity in a peptide-like arrangement, used in the following discussion is in order. Most of the sequences can be broadly classified into straight sequences containing molecules related by translation, zigzag sequences generated by 2_1 screw axes, and DL sequences generated by glide planes. A straight sequence is designated as S1 if the acceptor in the hydrogen bond is the carboxylate oxygen *cis* to the amino nitrogen atom and S2 if the acceptor atom is *trans* to it. The zigzag and DL sequences are correspondingly designated Z1 and Z2, and DL1 and DL2.

The amino acids for which the crystal structures of the L isomer as well as the racemate are available are valine, leucine, isoleucine, methionine, alanine, serine, tyrosine, histidine, glutamic acid and aspartic acid, and the analysis outlined below is based on these structures. The crystals of L-glutamic acid exist in two forms

rogen bonds involving main chain atoms. The sole exception is the α form of L-
amic acid (Lehmann and Nunes, 1980) in which the main chain atoms are
lved in a truly 3-dimensional network of hydrogen bonds. The 2-dimensional
ures are essentially of two types, one double-layers and the other, single layers.
tals of the hydrophobic amino acids L-valine (Torii and Iitaka, 1970), L-leucine
rding and Howieson, 1976), L-isoleucine (Torii and Iitaka, 1971) and L-
hionine (Torii and Iitaka, 1973) are made up of double-layers stacked along the
est crystallographic axis. As an example the crystal structure of L-valine is
vn in figure 2. Each layer in the double-layer has molecules held together by a
nd a S2 sequence. The arrangement of molecules in this layer is schematically
rated in figure 3. The layers in the double-layer are related by 2_1 screw axes
interconnected by N–H–––O hydrogen bonds. Thus the core of a double-layer
ade up of two sheets, each belonging to a layer. Each sheet is made up of main
n atoms and is stabilised by head-to-tail sequences. The two sheets are also
connected by N–H––O hydrogen bonds. The core is then flanked by side
ns.

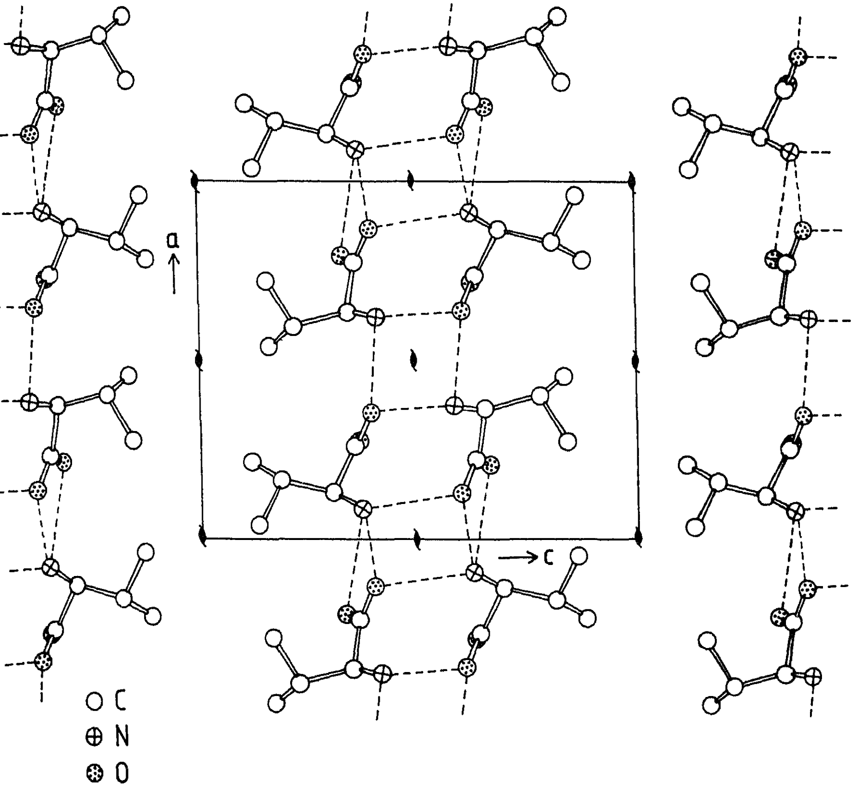

Figure 2. The crystal structure of L-valine as viewed along the *b* axis (see text for details).

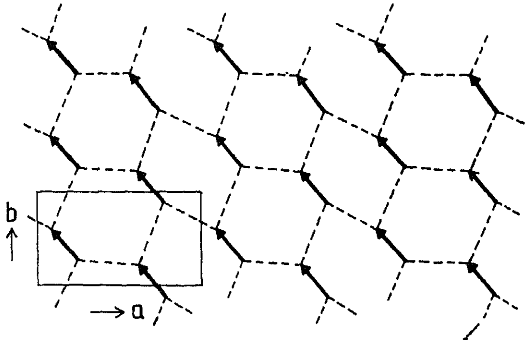

Figure 3. Schematic representation of the S1S2 arrangement in the layers of L-valine. In this and the following schematic diagrams, the head of an arrow represents the α-amino group, and the tail the α-carboxylate group, of an amino acid molecule.

The crystal structures of L-tyrosine (Mostad *et al.*, 1972; Frey *et al.*, 1973a), the two forms of L-histidine (Madden *et al.*, 1972a, b), the *β* form of L-glutamic acid (Hirokawa, 1955), L-aspartic acid (Derissen *et al.*, 1968), L-serine (Kistenmacher *et al.*, 1974), L-serine·H_2O (Frey *et al.*, 1973b) and L-alanine (Simpson and Marsh, 1966; Lehmann *et al.*, 1972) contain layers stacked along the longest axis. As an example, the structure of the monoclinic form of L-histidine is illustrated in figure 4. It may be noted that most of the amino acids mentioned above are hydrophilic in nature. Each layer consists of a central sheet made up of main chain atoms and stabilised by interactions involving α-amino and α-carboxylate groups. The central sheet is flanked on either side by side chains. Unlike in the case of the arrangement

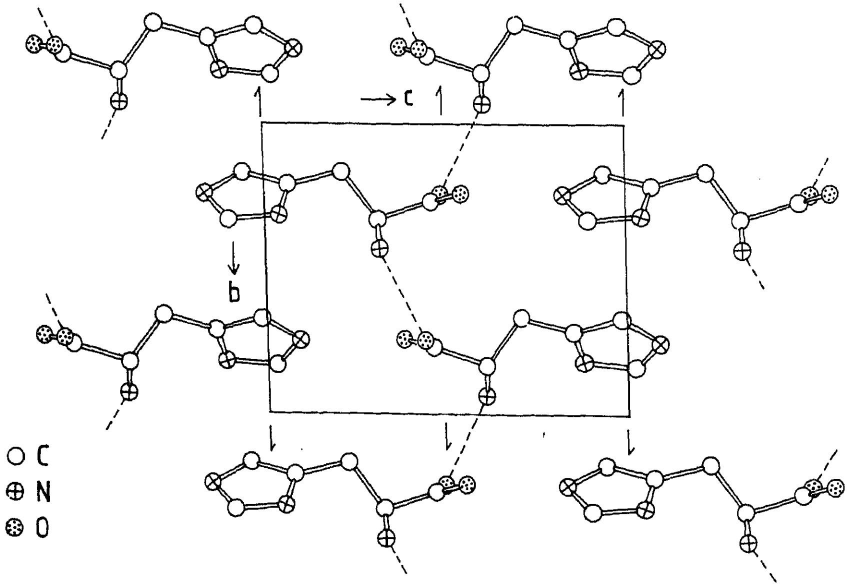

Figure 4. The crystal structure of L-histidine as viewed along the *a* axis (see text for details).

n chain-main chain interactions between adjacent layers except in the case of L- ine and L-serine. The small size of the side chains of these two amino acids nits interactions between adjacent sheets. In all the structures except that of L- ne·H_2O, the molecules in each layer are held together by a S2 sequence and a sequence. This S2Z2 arrangement in a layer is schematically illustrated in e 5. In the layers of L-serine·H_2O, however, a Z2Z2 arrangement, illustrated matically in figure 6 exists. Although the above discussion pertains to only e amino acids for which the crystal structures of both the L-isomer and the mate are available, it must be mentioned that the structures of other amino s also broadly follow the aggregation pattern outlined above. For example, the tal structures of the hydrophilic amino acids L-threonine (Shoemaker *et al.*,); Ramanadham *et al.*, 1973) and L-glutamine (Cochran and Penfold, 1952; tzle *et al.*, 1973) contain S2Z2 layers. L-Cysteine crystallises in two forms. The egation pattern in the orthorhombic form (Kerr and Ashmore, 1973, 1975) is d on layers while the monoclinic form (Harding and Long, 1968) has an egation pattern involving double-layers.

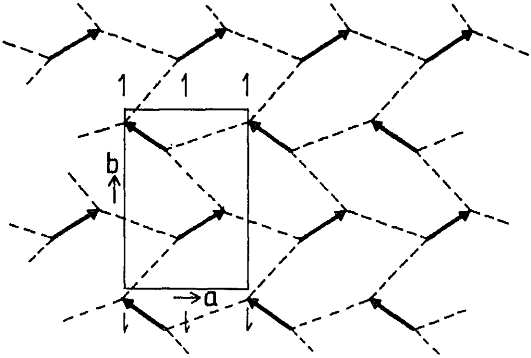

Figure 5. Schematic representation of a typical S2Z2 arrangement in the layers of most hydrophilic amino acids.

he reversal of chirality of half the molecules produces only minimal changes in aggregation patterns of the hydrophobic amino acids valine, leucine and eucine as seen by the examination of the crystal structures of DL-valine llikurjan and Thyagaraja Rao, 1969), DL-leucine (DiBlasio *et al.*, 1975) and isoleucine (Benedetti *et al.*, 1973). The molecules still aggregate into double- rs stacked along the longest crystallographic axis. In the L amino acid crystal ctures, both layers in the double-layer obviously contain the L isomer, and they related to each other by screw axes. In the DL amino acid crystal structures, layer contains only the L molecules and the other, the D molecules. Both the rs continue to have the S1S2 arrangement of head-to-tail sequences. The two rs in the double-layer are then related by inversion centres. The molecules in the tals of both α- and β-DL-methionine (Mathieson 1952) also aggregate into ble-layers as in the crystals of the other DL hydrophobic amino acids. However, ne structures of α- and β-DL-methionine, each layer in the double-layer contains

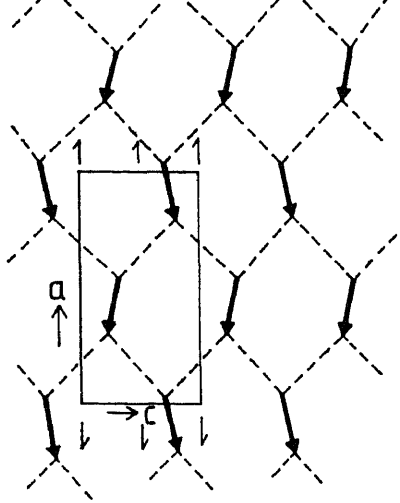

Figure 6. Schematic representation of the Z2Z2 arrangement in the layers of L-serine·H_2O.

L as well as D molecules (related by glide planes). The crystal structure of α-DL-methionine is shown in figure 7. The two layers in the double-layer are related to each other by screw axes as well as inversion centres. In each layer, the molecules are held together by two DL2 head-to-tail sequences, as schematically illustrated in figure 8.

A greater variety exists in the aggregation patterns observed in the crystal structures of DL-tyrosine (Mostad and Romming, 1973), DL-histidine (Edington and Harding, 1974), DL-glutamic acid (Ciunik and Glowiak, 1983), DL-aspartic acid (Rao, 1973), DL-serine (Kistenmacher *et al.*, 1974) and DL-alanine (Donohue, 1950). All of them contain single layers as do the crystals of the corresponding L isomers. The molecular arrangement in the L amino acid and the DL amino acid crystals are very similar in the case of alanine and tyrosine. Each layer in the DL amino acid crystals contains D as well as L molecules. The molecules in the layer are held together by a S2 head-to-tail sequence and a DL2 sequence. As far as the main chain atoms are concerned, the geometry of the DL2 sequence is close to the Z2 sequence in the crystals of the L isomers. Thus the S2Z2 arrangement in the L amino acid crystals and S2DL2 arrangements in the DL amino acid crystals are essentially similar. Consequently, the reversal in the chirality of half the molecules brings about only marginal changes in the essential features of the aggregation patterns in the case of tyrosine and alanine. A close relationship exists between the aggregation patterns in the crystals of L-serine·H_2O and in those of DL-serine. As in the case of L-serine·H_2O, a Z2Z2 pattern exists in the molecular layers in the crystals of DL-serine except that the alternating layers are of opposite chirality in the latter. The arrangement of molecules in the remaining 3 DL amino acid crystals, namely, those of DL-histidine, DL-aspartic acid and DL-glutamic acid, do not bear any relationship with one another, with those in other DL amino acid crystals or

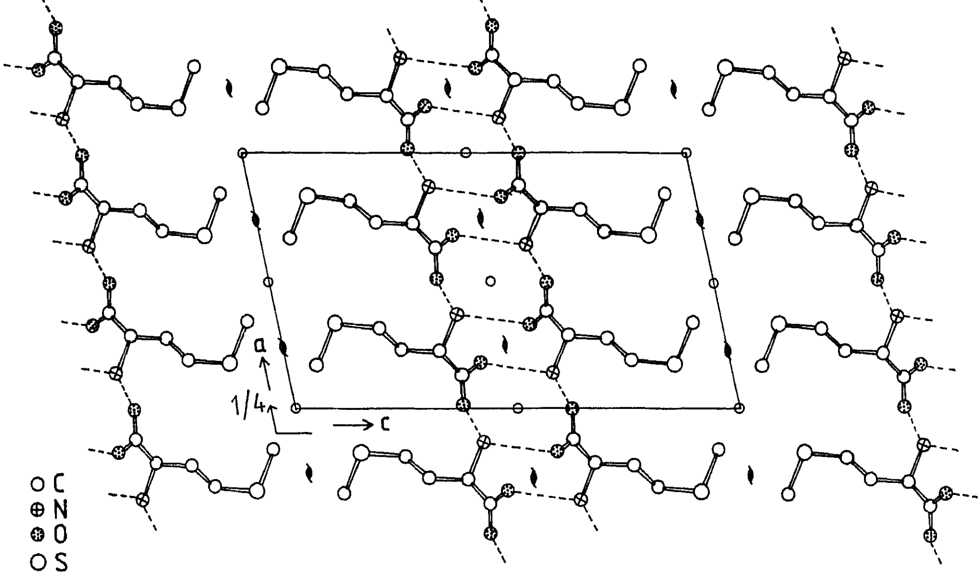

Figure 7. The crystal structure of α-DL-methionine as viewed along the *b* axis (see text for details).

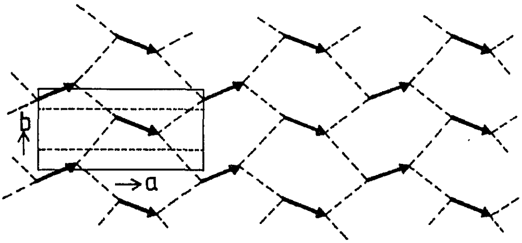

Figure 8. Schematic representation of the DL2DL2 arrangement in the layers of α-DL-methionine.

with those in the crystals of the corresponding L amino acids. The 3 arrangements are schematically illustrated in figure 9. A DL2 sequence is present in each of them. The DL-histidine layer consists of inversion centres but no other head-to-tail sequences. The DL-aspartic acid layers and the DL-glutamic acid layers contain in addition a Z2 sequence and a DL1 sequence respectively.

The broad basic feature of aggregation in most amino acid crystals is a layer or a double-layer. Mostly the same feature is found in a L amino acid crystal and the corresponding DL amino acid crystal. The arrangement of molecules in a layer in a L amino acid crystal and that in the corresponding DL amino acid crystal are found to be the same or closely related in a majority of the cases. These arrangements are substantially different only in 3 cases. Thus when all the amino acid molecules in a crystal are of the same chemical type, the general patterns of molecular aggregation tend to be retained even when the chirality of half the molecules is reversed. It however turns out that the packing efficiency in the crystals

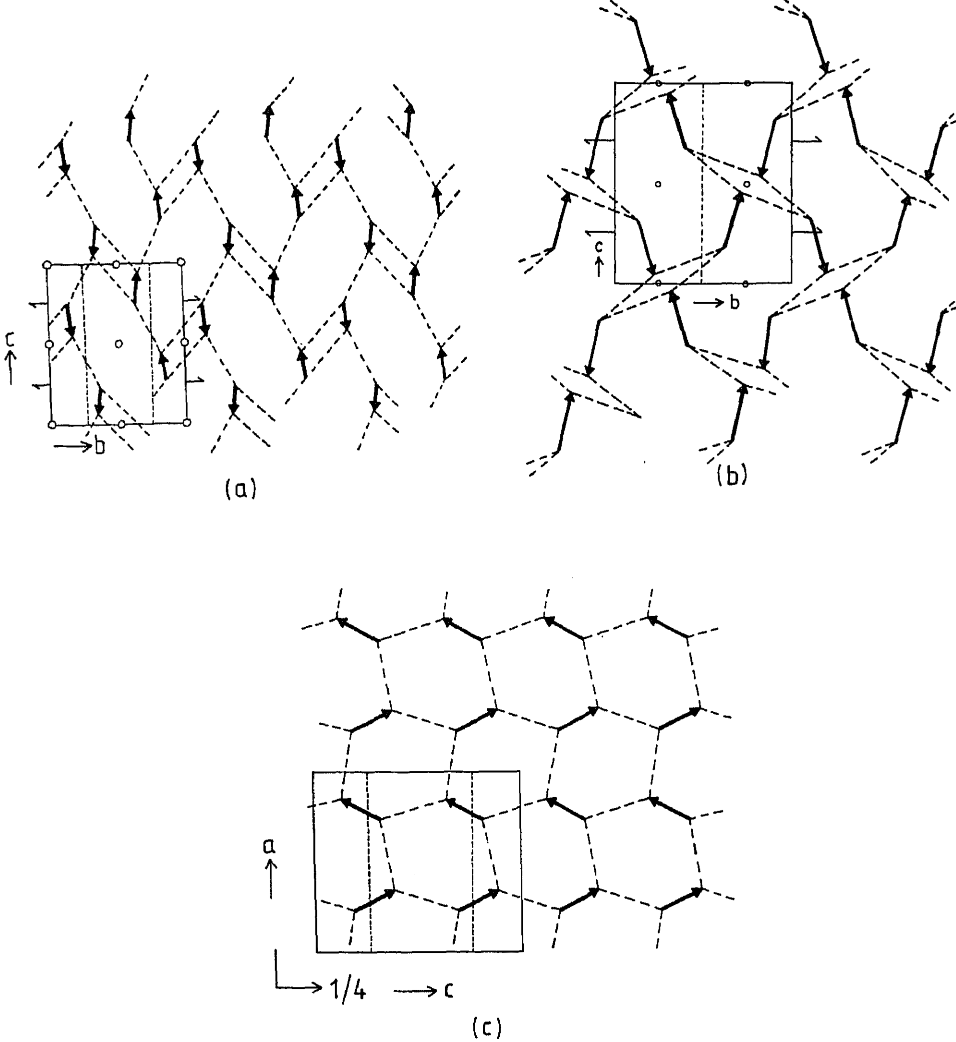

Figure 9. Schematic representation of (a) the arrangement consisting of a DL2 sequence and inversion centres in the layers of DL-histidine, (b) the DL2Z2 arrangement in the layers of DL-aspartic acid and (c) the DL1DL2 arrangement in the layers of DL-glutamic acid.

of DL amino acids is in general slightly higher than that in the crystals of L amino acids. In all but one case, the unit cell volume per molecule is slightly lower in the former than in the latter. The percentage difference in this volume ranges between 0·1 and 5·9.

LL and LD complexes: There are 4 amino acid-amino acid complexes for which the LL and LD type crystal structures are available. They are lysine aspartate (Bhat and Vijayan, 1976; Soman *et al.*, 1988), arginine glutamate (Bhat and Vijayan, 1977; Suresh *et al.*, 1986), arginine aspartate (Salunke and Vijayan, 1982; Suresh *et al.*,

erms of layers. In the 5 remaining LL complexes, the crystal structures
dered as made up of alternating layers of basic and acidic molecules.
s in the basic layer in L-lysine L-aspartate and in L-arginine L-
e a S1Z2 arrangement which is similar to that found in the layers in
st hydrophilic amino acids (figure 5). A Z2Z2 arrangement similar to
the crystals of L-serine·H_2O exists in the basic layer of L-arginine L-
nohydrate. In the acidic layers in the LL complexes, head-to-tail
hemselves do not define a plane. Other interactions are also necessary
The aspartate layer in L-lysine L-aspartate and L-arginine L-aspartate
one Z1 type head-to-tail sequence parallel to the shortest repeat
glutamate layer in L-arginine L-glutamate contains two Z1 sequences
e shortest repeat distance, but they also stabilise only a column of
her interactions are needed to define the layer.

t differences between the LL and the corresponding LD complexes
ase of the complexes involving arginine. In L-arginine D-glutamate
d L-arginine D-glutamate monohydrate (reported in this paper) the
ange themselves in double-layers in a fashion similar to that found in
of α-DL-methionine illustrated in figure 7. Incidentally, a similar
exists in the structure of L-lysine D-glutamate also. However, as L-
mate could not be crystallised so far, this arrangement cannot be
h that in the corresponding LL complex. An arrangement based on
exists in the crystal structure of L-arginine D-aspartate also. Each
ouble layer contains two S1, DL1 and DL2 sequences. In contrast to
encountered in L-arginine D-glutamate and L-arginine D-aspartate
D-glutamate), unlike molecules aggregate into separate alternating
structure of L-lysine D-aspartate monohydrate. The arrangement of
he lysine layer is the same as that found in L-lysine L-aspartate. The
of molecules in the aspartate layer, however, is different from that in
lex. In fact no head-to-tail sequence exists in this layer. The crystal
L-ornithine D-aspartate monohydrate is also based on alternating
rangement in the ornithine layer is similar to that found in the lysine
ysine aspartate complexes. However, the arrangement in the aspartate
nvolves a S2 head-to-tail sequence, is different from any observed so
structures containing aspartic acid or aspartate ions. It must also be
at the aggregation pattern in L-ornithine D-aspartate monohydrate
nblance to that in the corresponding LL complex.

from the above discussion that the basic elements of molecular
ound in the complexes are by and large similar to those observed in
f L and DL amino acids. However, the changes brought about in the
the distribution of these elements when going from LL to the
g LD complexes are often more drastic than the differences between
f L amino acids and those of the corresponding DL amino acids. For
LL complexes involving arginine have alternating layers of unlike
the basic pattern of aggregation whereas the corresponding LD
e made up of double-layers. In the case of lysine aspartate, both the
D complexes contain alternating layers of basic and acidic molecules.

The aggregation of molecules in the lysine layer is similar in the two complexes, but it is entirely different in the aspartate layer. As far as ornithine aspartate is concerned, the LL and the LD complexes have fundamentally different aggregation patterns. Thus while the reversal of chirality of half the molecules in crystals made up of amino acids of the same type leads only to comparatively small differences in the aggregation pattern in most cases, profound changes result when the chirality of one type of molecules is reversed in crystals composed of equal number of basic and acidic amino acid molecules. These changes could, however, be of many types.

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A new radioactive diazofluorene based heterobifunctional reagent

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Abstract. Synthesis of a radioactive photoactivable heterobifunctional reagent, N-oxysuccinimide ester of 2-[^{14}C]glycyl carboxy-9-diazofluorene is described. This reagent on photolysis gives rise to a reactive carbene which rapidly inserts into solvents like methanol. The probe can be easily linked to aldolase which on photolysis gives rise to aldolase dimer, trimer and tetramer depending on the density of linked probe. This probe has also been linked to concanavalin A. The radioactive concanavalin A so obtained was incubated with erythrocyte ghosts and photolysed. The membrane protein analysis by gel electrophoresis indicated that concanavalin A has been covalently crosslinked to band 3.

Keywords. Photoactivable; heterobifunctional reagent; carbene; diazofluorene; aldolase; concanavalin A-band 3.

Introduction

Photoaffinity labelling has proved to be a powerful technique for studying biomolecular interactions (Bayley, 1984). Its use in identification of several receptor proteins has been well documented (Eberle and DeGran, 1985). This application involves the use of photoactivable heterobifunctional reagents, which incorporate a chemically reactive and a photochemically reactive site. The photoactivable site is usually a nitrene precursor *e.g.* aryl azides and several reagents belonging to this category have been reported (Ji, 1983). Despite the documented advantages of carbene precursors as photoactivable reagents (Bayley and Knowles, 1978; Brunner and Semenza, 1981) and their extensive use *e.g.* in labelling membrane bound proteins, very few carbene based heterobifunctional reagents have been reported (Nassal, 1983). We have recently reported N-oxysuccinimide ester of 2-carboxy-9-diazofluorene (NOH-CAB-DAF) as one such reagent (Mogre *et al.*, 1987). We report here a radioactive analogue of NOH-CAB-DAF which provides a sensitive method of monitoring and gives rise to aldolase crosslinking data which is similar to that obtained with NOH-CAB-DAF. Further, concanavalin A (ConA) was modified with this new reagent and used to study its crosslinking with band 3.

Materials and methods

All chemicals and solvents were commercial grades of highest purity and were further purified if required according to Perrin *et al.* (1980). UV-visible spectra were recorded on Shimadzu UV-260 spectrophotometer. High performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-4A liquid chromatograph using Shimpak CLC ODS column (6 × 150 mm) and methanol: water (80:20 v/v) as a mobile phase at a flow rate of 1 ml/min. All HPLC analyses were carried out under these conditions unless specified otherwise. Eluent was usually monitored at 230 nm where all fluorenyl compounds could be detected while monitoring at 350 nm detected only the diazo compounds as diazofluorene (DAF) and its analogues reported here absorb strongly around 350 nm. NOH-CAB-DAF was prepared from 2-carboxy-9-DAF as reported earlier (Mogre *et al.*, 1987). [^{14}C]Glycine of specific activity 21·4 mCi/mmol was purchased from Bhabha Atomic Research Centre, Bombay. Radioactivity was determined on a LKB 1217 Rackbeta liquid scintillation counter using a toluene based scintillant (0·4% PPO, 0·025% dimethyl-POPOP). Photolysis was performed on Rayonet miniphotochemical reactor RMR-500 fitted with four 3500 Å lamps. Care was exercised in handling unphotolysed material and all operations were carried out using safe brown light. Samples were purged with a stream of nitrogen prior to photolysis. Aldolase type IV and ConA were purchased from Sigma Chemical Co., Missouri, USA. Crystalline suspension of rabbit muscle aldolase in 2·5 M $(NH_4)_2SO_4$ was extensively dialysed against 5 mM sodium phosphate buffer pH 7·4. The final concentration of aldolase was determined by absorbance at 280 nm, $E^{1\%}_{280} = 9{\cdot}38$ (Donavan, 1964). Erythrocyte ghosts were prepared according to Steck and Kant (1974). Ghosts protein concentration was determined by modified Lowry procedure (Markwell *et al.*, 1981). Electrophoresis was performed according to Fairbanks system (Fairbanks *et al.*, 1971). Samples were applied to a 4% polyacrylamide gel rods and run at a constant current of 3 mA/gel. After electrophoresis the radioactive gels were cut into 2 mm slices. The gel slices were then solubilized in 10 μl of water and 200 μl of Luma Solve (Lumac Systems) for 12 h and counted in toluene based scintillation fluid.

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Abbreviations used: NOH-CAB-DAF, N-Oxysuccinimide ester of 2-carboxy-9-diazofluorene; ConA, concanavalin A; HPLC, high performance liquid chromatography; DAF, diazofluorene; NOH-[^{14}C]GLY-DAF, N-oxysuccinimide ester of 2-[^{14}C]glycylcarboxy-9-diazofluorene; R_t, retention time; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; M_r, molecular weight; PPO, diphenyl oxazole; POPOP, 1,4-bis-(5-phenyl oxazol-2-yl) benzene.

NOH-[^{14}C]GLY-DAF

NOH-CAB-DAF (2·1 mg, 6 μmol) was dissolved in dry dioxan (0·5 ml) and aqueous sodium bicarbonate (2 μl of a 1·2 M solution) was added. It was then stirred with [^{14}C]glycine (25 μCi, 1·17 μmol) for 5 h at room temperature, poured into ice-cold water (10 ml) and carefully acidified with 0·05 N acetic acid (100 μl) at 0°C. It was then extracted with CH_2Cl_2. The solvent was removed with a stream of nitrogen and the residue was taken in dry dioxan (0·5 ml). The UV-visible spectrum showed bands at 242, 305 and 372 nm, as observed in the case of authentic 2-glycylcarboxy-9-DAF. The radioisotope HPLC analysis was carried out at this stage and 1 ml fractions were collected. These fractions were counted on a liquid scintillation counter using toluene based scintillation fluid. The radioactivity elution profile derived from the counts in each fraction indicated that more than 80% of [^{14}C]glycylcarboxy-9-DAF is formed based on the retention time (R_t) of 2-glycylcarboxy-9-DAF ($R_t = 5{\cdot}1$ min).,

To the above dioxan solution (0·5 ml), N-hydroxy succinimide (0·8 mg, 6 μmol) and dicyclohexylcarbodiimide (1·2 mg, 6 μmol) were added. It was then stirred for 20 h at room temperature. The radio HPLC analysis was again carried out at this stage as before. The radioactivity elution profile derived from the counts in each fraction indicated that more than 60% of the parent [^{14}C]glycine condensation product has been converted to the N-oxysuccinimide ester based on the R_t of authentic N-oxysuccinimide ester of 2-glycylcarboxy-9-DAF (R_t = 10·6 min). The UV-visible spectrum showed bands at 250, 301 and 372 nm as observed in N-oxysuccinimide ester of 2-glycylcarboxy-9-DAF. This material could be easily purified further by HPLC and the specific activity of the material was found to be 21·1 mCi/mmol. The crosslinking experiments could also be carried out without purification by HPLC as at this stage the final reagents purity is 60% and on incubation with aldolase only the N-oxysuccinimide ester *i.e.* NOH-[^{14}C]-GLY-DAF is linked whereas the residual unreacted [^{14}C]GLY-DAF is removed during gel permeation chromatography on Sephadex G-25. Similar crosslinking of aldolase was observed even with 60% pure reagent.

Crosslinking of aldolase using NOH-[^{14}C]GLY-DAF

Aldolase (0·04 nmol, 0·133 μM) in 5 mM phosphate buffer pH 7·4 containing 0·1 M $NaHCO_3$, was treated in dark with 7·5 (7·4 nCi) and 37·5 (31·8 nCi) fold molar excess of NOH-[^{14}C]GLY-DAF. The probe was added as a dioxan solution, 1 and 5 μl respectively, to 0·3 ml of aldolase solution. The samples were then incubated for 1 h at room temperature and then applied to a Sephadex G-25 column and eluted with 50 mM phosphate buffer pH 7·4. Fractions appearing in the void volume were collected and photolysed for 3 min and analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Chemical modification of ConA with NOH-[^{14}C]GLY-DAF and its crosslinking to erythrocyte ghosts

A aliquot (1 μCi) from dioxan solution of the probe was added to 10·2 μg (0·4 nmol) of ConA in 5 mM phosphate buffer, pH 7·4 containing 0·1 M $NaHCO_3$ so that the final pH was 8·6. The probe to protein molar ratio was 1:7·5 and final dioxan concentration was 3·3% v/v. After incubation for 2 h the whole solution was subjected to chromatography on a Sephadex G-25 column. Erythrocyte ghosts were equilibrated with 50 mM phosphate buffer, pH 7·4. The material appearing in the void volume, which corresponds to probe linked ConA, was added to erythrocyte ghosts (0·2 mg protein). After incubation for 30 min the whole preparation was photolysed for 3 min. It was then centrifuged at 15000 *g* for 30 min, washed with 5 mM phosphate buffer, pH 7·4 and subjected to SDS-PAGE(4%).

Results and discussion

The radioactive reagent, NOH-[^{14}C]GLY-DAF was prepared from NOH-CAB-DAF. The reagent was analysed by HPLC and was found to be identical to authentic NOH-GLY-DAF. The detailed synthesis and characterization of NOH-GLY-DAF will be reported elsewhere. The final reagent could be purified by HPLC and had a specific activity of 21·1 mCi/mmol. The UV-visible spectrum of NOH-[^{14}C]GLY-DAF is given in figure 1. NOH-[^{14}C]GLY-DAF is highly sensitive to light and heat. It should be handled carefully in a dark room and stored at −20°C

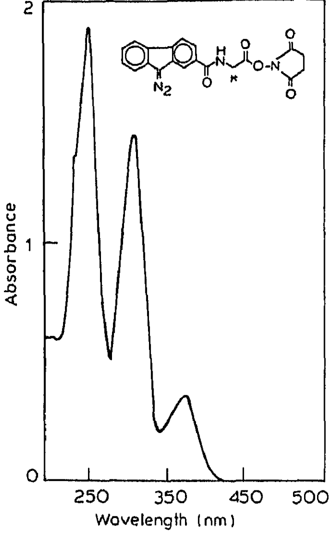

Figure 1. UV-visible spectrum of NOH-[^{14}C]GLY-DAF.

in dry dioxan. Interestingly the HPLC analysis indicated that it is not always the photochemical degradation, but the chemical degradation of probes which predominates. Thus the N-oxysuccinimide ester group in both NOH-CAB-DAF ($R_t = 11{\cdot}03$ min) and NOH-GLY-DAF ($R_t = 10{\cdot}6$ min) hydrolyse in methanol solution at 25°C to the parent acids, 2-carboxy-9-DAF ($R_t = 5{\cdot}4$ min) and 2-glycylcarboxy-9-DAF ($R_t = 5{\cdot}1$ min). This hydrolysis in methanol takes place only if methanol contains traces of water. The hydrolysis is a slow reaction and approximately 10% hydrolysis per day was observed in a sample stored at 4°C in dark. The diazo group did not undergo decomposition under these conditions as estabilished by monitoring the eluant at 350 nm. The normal HPLC eluent is monitored at 230 nm but monitoring at 350 nm gives peaks only if the parent DAF moiety is intact. In short, solutions of NOH-[^{14}C]GLY-DAF in dioxan or methanol should be prepared in anhydrous solvents taking care that moisture is not absorbed during preparation of the solution.

Crosslinking of aldolase using NOH-[^{14}C]GLY-DAF

The NOH-[^{14}C]GLY-DAF was linked to aldolase at molar ratio of 1:7·5 and 1:37·5. The unlinked probe was removed by gel permeation chromatography. The modified aldolase was then photolysed and analysed by SDS-PAGE (figure 2). At the lower molar ratio one observes largely the monomer along with small amounts of dimer, trimer and tetramer (figure 2A), whereas at higher molar ratio tetramer appears as the major band (figure 2B). Due to lower resolution of tetramer and higher oligomers of aldolase on SDS-PAGE(4%), Coomassie blue staining of aldolase crosslinked with NOH-CAB-DAF gives rise to a blur on top of the gel (Mogre *et al.*, 1987). The use of [^{14}C] labelled probe, NOH-[^{14}C]GLY-DAF permits a more clearer analysis indicating that higher oligomers are formed but the

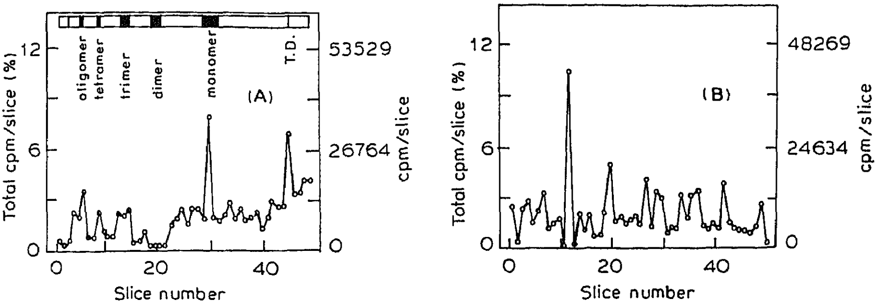

Figure 2. Photochemical crosslinking of aldolase modified with NOH-[^{14}C]GLY-DAF. Aldolase was linked to this probe by adding 7·5 and 37·5 molar excess of NOH-[^{14}C]GLY-DAF, the unlinked probe being removed by chromatography on Sephadex G-25. The material appearing in the void volume was photolysed and subjected to SDS-PAGE (4%). These gels were then cut into 2 mm slices and counted. (A), Lower probe concentration gel. (B), Higher probe concentration gel. The gel shown on top of (A) is a schematic of parallel gel run with crosslinked aldolase and stained with Coomassie blue.

tetramer is the major band, as expected from the native tetrameric state of rabbit muscle aldolase.

Rabbit muscle aldolase has been used in the past as a model protein for studying crosslinking. It normally exists as a tetramer comprising of 4 subunits with a M_r of 40000. Recently thiol specific fluorogenic heterobifunctional reagents based on azido coumarins have been used for crosslinking of aldolase (Ueno *et al.*, 1984). Using this reagent one observes largely the formation of aldolase dimer and barely observable trimer or tetramer. The use of NOH-CAB-DAF reported by us earlier (Mogre *et al.*, 1987) and the radioactive analogue reported here, NOH-[^{14}C]GLY-DAF, gives rise to a much higher degree of crosslinking possibly as a result of higher reactivity of carbenes.

Crosslinking studies of NOH-[^{14}C]GLY-DAF modified ConA with erythrocyte ghosts

ConA was modified with NOH-[^{14}C]GLY-DAF at 1:7·5 molar ratio and subjected to gel permeation chromatography to remove the unlinked probe. The modified ConA was incubated with erythrocyte ghosts and photolysed. The SDS-PAGE (figure 3) showed both ConA monomer and dimer with the latter appearing as the major peak. In addition one observes two small peaks corresponding to M_r 120,000 and 142,000 Da and some high M_r material running at the top of the gel. The 120,000 and 142,000 Da bands correspond to expected M_r of ConA-band 3 and ConA dimer-band 3 crosslinked products.

The lectin ConA is known to bind to erythrocytes and we chose this as a model system to assess the potential of our probe as this system has been investigated earlier using phenyl azide based heterobifunctional reagents *i.e.* 4-azidobenzoimidate (Ji, 1977) and N-oxysuccinimide ester of 4-azidosalicylic acid (Ji and Ji 1982). In both these cases a band corresponding to ConA dimer and a band which barely moved in the SDS-polyacrylamide gel was refered to as ConA crosslinked to its receptor, possibly band 3. Using NOH-[^{14}C]GLY-DAF modified ConA we also observed the ConA dimer observed by Ji (1977) and Ji and Ji (1982). In addition

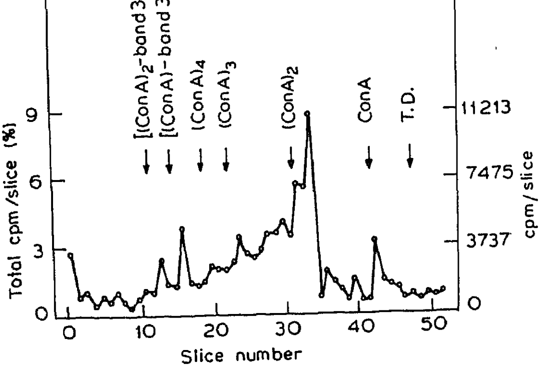

Figure 3. Photochemical crosslinking of ConA modified with NOH-[^{14}C]GLY-DAF to erythrocyte ghosts. The expected position of bands correspond to M_r of ConA monomer, dimer-(ConA)$_2$, trimer-(ConA)$_3$, tetramer-(ConA)$_4$, ConA crosslinked to band 3-[ConA-band 3] are marked on top. These R_f values were derived on the basis of semilog plot of standard M_r marker proteins run on a parallel gel and taking ConA monomer M_r as 25,500 and band 3 as 97,000.

two bands corresponding to M_r of crosslinked ConA-band 3 and ConA dimer-band 3 were observed besides some material which barely enters the gel (figure 3). These results clearly indicate that ConA is crosslinked to band 3 in erythrocytes. Further studies can be carried out to comment further on the nature of ConA-band 3 interactions in erythrocytes.

We will finally like to conclude that high reactivity of this reagent and the availability of the radioactive preparation should make this reagent very useful in protein crosslinking studies.

Acknowledgement

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Hydrophilicity and antigenicity of proteins—A case study of myoglobin and hemoglobin

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Abstract. Hydrophilicity index is used to locate antigenic determinants on two related groups of proteins—myoglobin and hemoglobin. The data on 41 species (including 34 mammals) of myoglobin show that average hydrophilicity for the complete myoglobin molecules as well as the average hydrophilicity for all hydrophilic regions put together seem to remain constant; the variation in the size and location of the antigenic determinants in these species is very small indicating that the antigenic sites are not shifted during evolution. In the case of both the proteins there is a good agreement between the antigenic sites picked up by using hydrophilicity index and the experimentally determined antigenic sites. The data on 56 species of hemoglobin α-chains and 44 species of hemoglobin β-chains showed that although there are few sites on hemoglobin which have remained invariant during evolution, there is a significant variation in other sites in terms of either a splitting of a site, or a drastic change in the hydrophilicity values and/or a length of the site. Comparison of the hydrophilicity data on these two groups of proteins suggests that hemoglobins which perform a variety of functions as compared to myoglobins are evolving faster than myoglobins supporting the contention of earlier workers.

Keywords. Hydrophilicity; antigenic sites; myoglobin; hemoglobin; evolution.

Introduction

Immunological properties of proteins have been used widely to study their structure. However, determination of complete immunogenic structure of a single protein from a given species is not very easy and some times takes a long time. In fact, there are only a few proteins such as sperm whale myoglobin (Atassi, 1975), hemoglobin (Kazim and Atassi, 1980, 1982) and lysozyme (Atassi, 1978) for which complete immunogenic structure is available. The experimental studies on proteins, particularly on lysozyme, have also pointed out that antigenic sites on proteins may be formed either by sequential continuous regions or by bringing together several antigenic determinants to form antigenic sites. The elaborate experimental procedures used for these studies and the time spent on them have necessitated the development of a theoretical approach for the prediction of immunogenic structure of a protein. One of the approaches which seems to have high potential to delineate antigenic determinants of the protein molecule is by Hopp and Woods (1981) and by Fraga (1982). This approach was recently used to predict and confirm the antigenic determinants of proteins such as RNAase A and seminalplasmin (Pandit, 1985). The success of this approach prompted us to apply the method to the proteins for which complete sequences are known for large number of species and at least in one case where both the 3-dimensional structure as well as complete immunogenic structure is known. The natural choice for our case study was therefore myoglobin and hemoglobin. The purpose of choosing these proteins was to examine the antigenic sites on functionally related groups of proteins and to see if it is possible to throw some light on the differences between myoglobins and hemoglobins as hemoglobin is involved in much more diversified functions than myoglobin. These studies showed that indeed the level of confidence in the prediction of antigenic sites can be increased substantially if, instead of applying the approach of Hopp and Woods (1981) to a single protein, it is applied to a group of related proteins of known sequences. The results obtained from the analysis of such related protein sequences are discussed along with their significance in the succeeding sections of this paper.

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Materials and methods

Amino acid sequences of myoglobin from 41 species (34 mammals, 5 reptiles and 2 birds) were taken from protein sequence data bank of NBRF. The species for which myoglobin sequences were analysed are given in figure 1. All the myoglobin sequences were arranged to get maximum homology among their sequences as has been done earlier by Hunt *et al.* (1978). The system of numbering the sequence after alignment was the same as followed by Hunt *et al.* (1978). This sequence data was further used to determine the hydrophilic regions on proteins. The algorithm which is quite similar to the one used by Hopp and Woods (1981, 1983) and Pandit (1985) to determine the hydrophilic regions is briefly discussed below. For each overlapping hexapeptide a profile of hydrophilic values as a function of the position in the sequence of the first amino acid of the hexapeptide is constructed using a computer program. Hydrophilicity values used are those given by Levitt (1976) with the adjustments suggested by Hopp and Woods (1981). Each hexapeptide was addressed by the position of the first amino acid in a hexapeptide sequence. Whenever the hydrophilicity values of at least 4 consecutive overlapping hexapeptides as well as the average hydrophilicity of all the amino acids consisting the region composed of such hexapeptides was greater than or equal to zero, such a region was picked up as the most probable antigenic site. The search was continued for delineating antigenic determinants for the entire sequence. This algorithm was applied to the sequences of myoglobin and hemoglobin of the species mentioned in figures 1 and 3 respectively, and antigenic determinants were determined.

Results and discussion

Probable antigenic sites along with their average hydrophilicity values for myoglobin sequences for all the species are given in figure 1. It can be seen from figure 1 that in case of myoglobin, 7 probable antigenic sites were obtained. These sites along with the experimentally observed antigenic sites for human myoglobin as reported by Atassi (1975) and Westhoff *et al.* (1984) are given in table 1. It can be seen from figure 1 and table 1 that all experimentally observed sites are picked up by our method except the site 166–172. The only theoretically predicted additional antigenic determinant is 155–166. In all other cases, however, one finds a very good

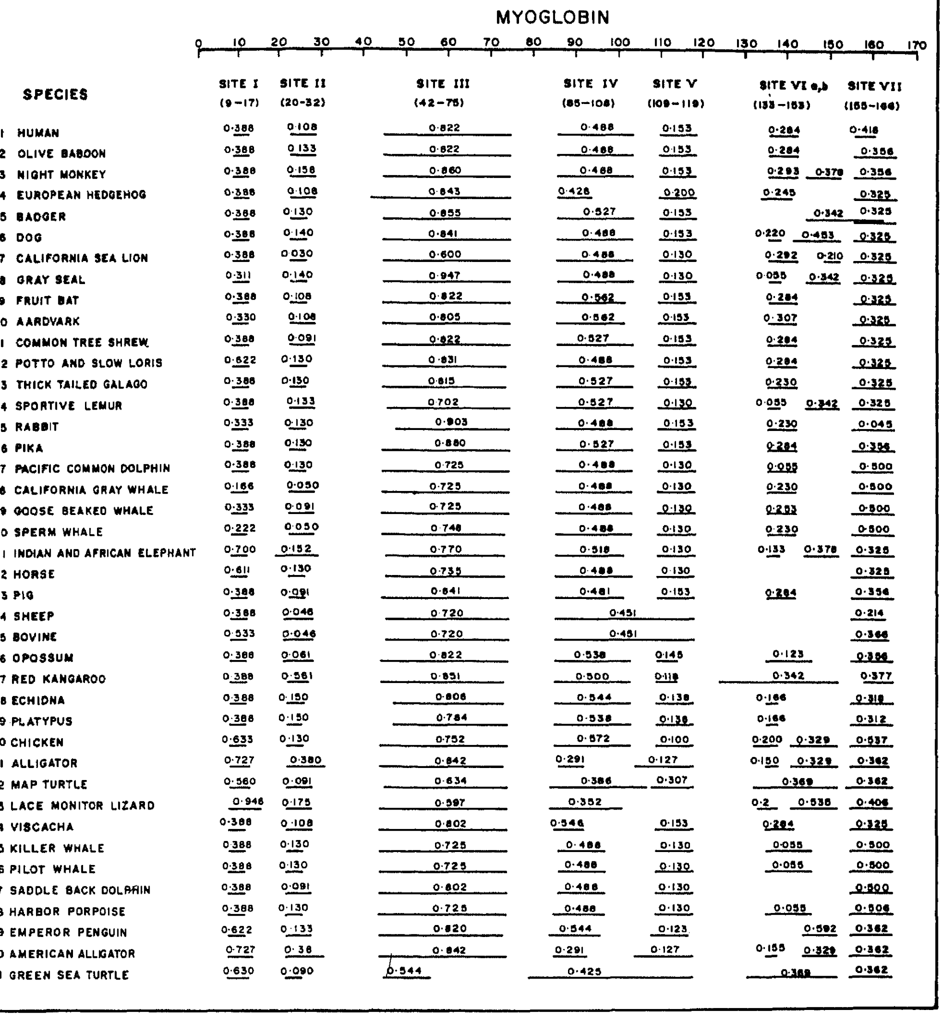


Figure 1. Hydrophilic sites on myoglobin from various species (the average hydrophilicity values of individual sites are given along with the sites).

greement between experimentally observed and theoretically predicted antigenic tes. It can also be seen that many of the sites such as sites I to IV and VII are resent in all the species without exception and there is a very little variation in the ngth of these sites. A close scrutiny of the hydrophilicity values indicates that the alues for sites II and V are not very high when compared with the hydrophilicity f the other sites. In fact, according to the rationale established by Hopp and Voods (1981, 1983) a good correlation between hydrophilicity value and antigenic te exists only for those two or three regions which have highest hydrophilicity

Table 1. Comparison of sites obtained on myoglobin by using average hydrophilicity values with the sites observed experimentally by earlier workers.

Site No.	Site based on hydrophilicity[a]	Experimentally observed sites	
		Atassi (1975)	Westhoff *et al.* (1984)
I	9– 17	—	9– 15
II	20– 32	24– 31	24– 31
III[b]	42– 68	—	31– 68
	69– 75	69– 74	69– 74
IV	85–108	—	85–108
V	109–119[c]	113–121	113–120
VI	133–147[d]	134–141	134–140
	142–153	—	142–148
VII	155–166	—	—
VIII	—	166–172	166–172

[a] Sites are depicted by the range covered by various species given in figure 1.
[b] Picked up as single site but shown separately for comparison.
[c] Except in case of alligator where the site was 105–119.
[d] Note a small overlap between the subsites of the site VI.

this study points out that the antigenic sites with relatively low hydrophilicity values (such as sites II and V) can be picked up if one carries out analysis similar to the one mentioned above on a similar protein from different species. It is interesting to note from the myoglobin data that although the order of the sequence-homology among proteins from various animals which are evolutionarily distant is less than 50%, the variation in the size and the location of antigenic determinants in these species is very small indicating that the antigenic sites are not shifted during evolution.

The quantitative variation in the average hydrophilicity values of different sites on myoglobin for all species studied is given in figure 2. A closer look at these values in few cases indicates that for a given species the change in the hydrophilicity value of one of the antigenic sites is associated with a compensatory change in the hydrophilicity value of the other site (ex. compare the hydrophilicity values of sites III and VII for species No. 15). Although one cannot generalize in regard to such a behaviour, this suggests that the delicate balance of the total hydrophilicity value for antigenic sites is maintained from one species to another species. This is further supported by the finding that the average hydrophilicity for complete myoglobin molecules as well as the average hydrophilicity values for all hydrophilic regions put together (see two lowermost profiles in figure 2) seem to remain constant. The maintenance of the above mentioned delicate balance of hydrophilicity of the protein molecule may be of a general nature and is probably necessary for an intrinsic functional requirement of the protein.

Hydrophilicity data obtained for hemoglobin species are given in figures 3 and 4. Kazim and Atassi (1980, 1982) have reported the antigenic sites on the α-chains of hemoglobins and few other species (table 2) by using synthetic approach in their confirmation of antigenic sites on these proteins. Comparison of the sites obtained by them with the sites picked up by using hydrophilicity values is given in table 2. It can be seen from the results that there is an appreciable amount of overlap between the experimentally confirmed antigenic sites in the species mentioned and

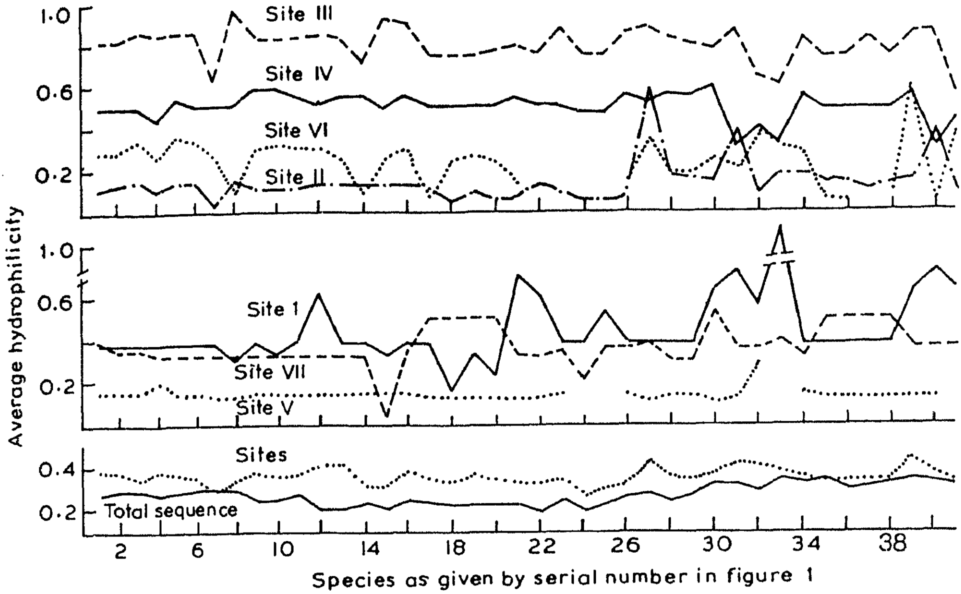
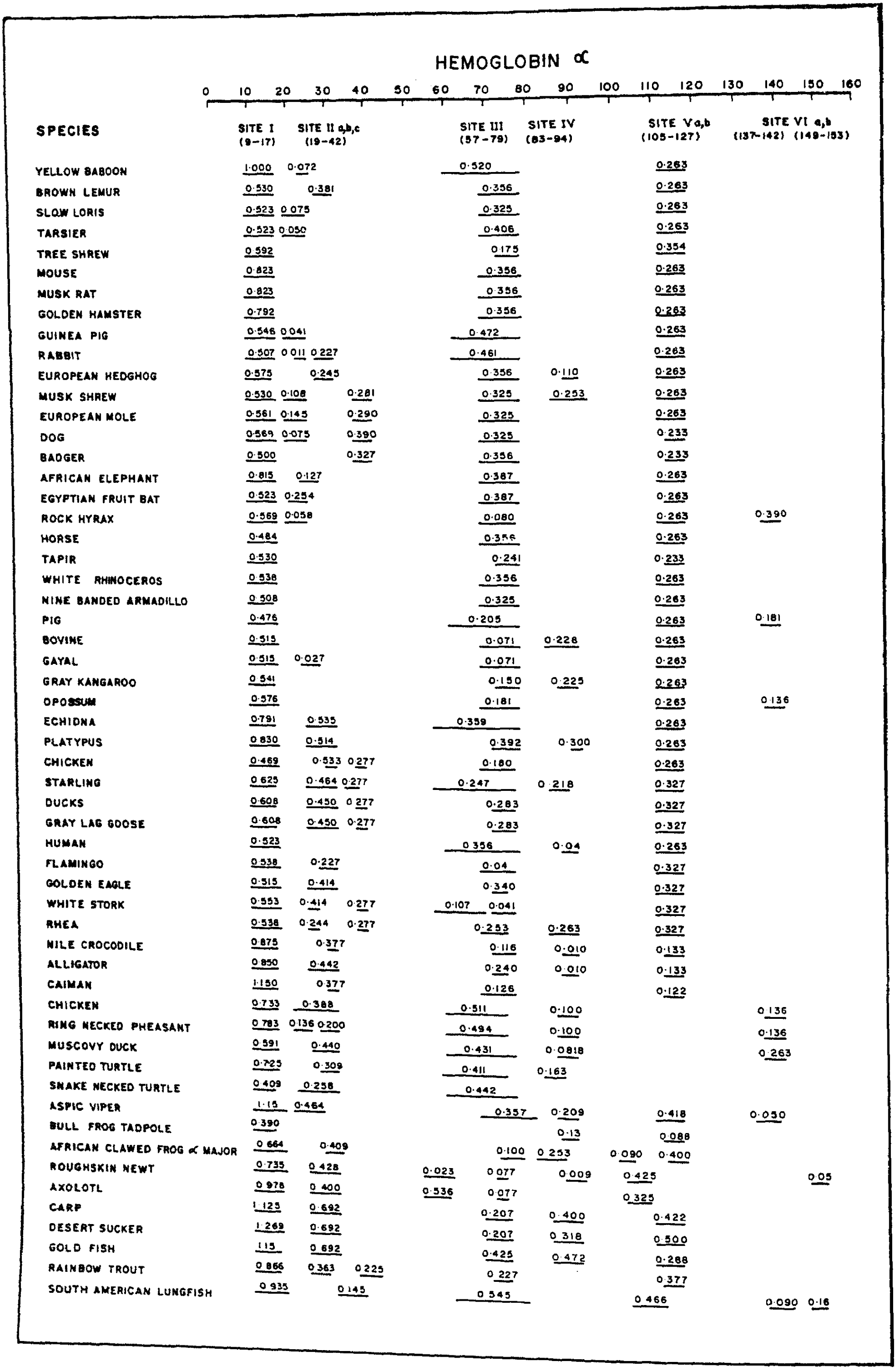


Figure 2. Average hydrophilicity values for different sites on the myoglobins from various species.

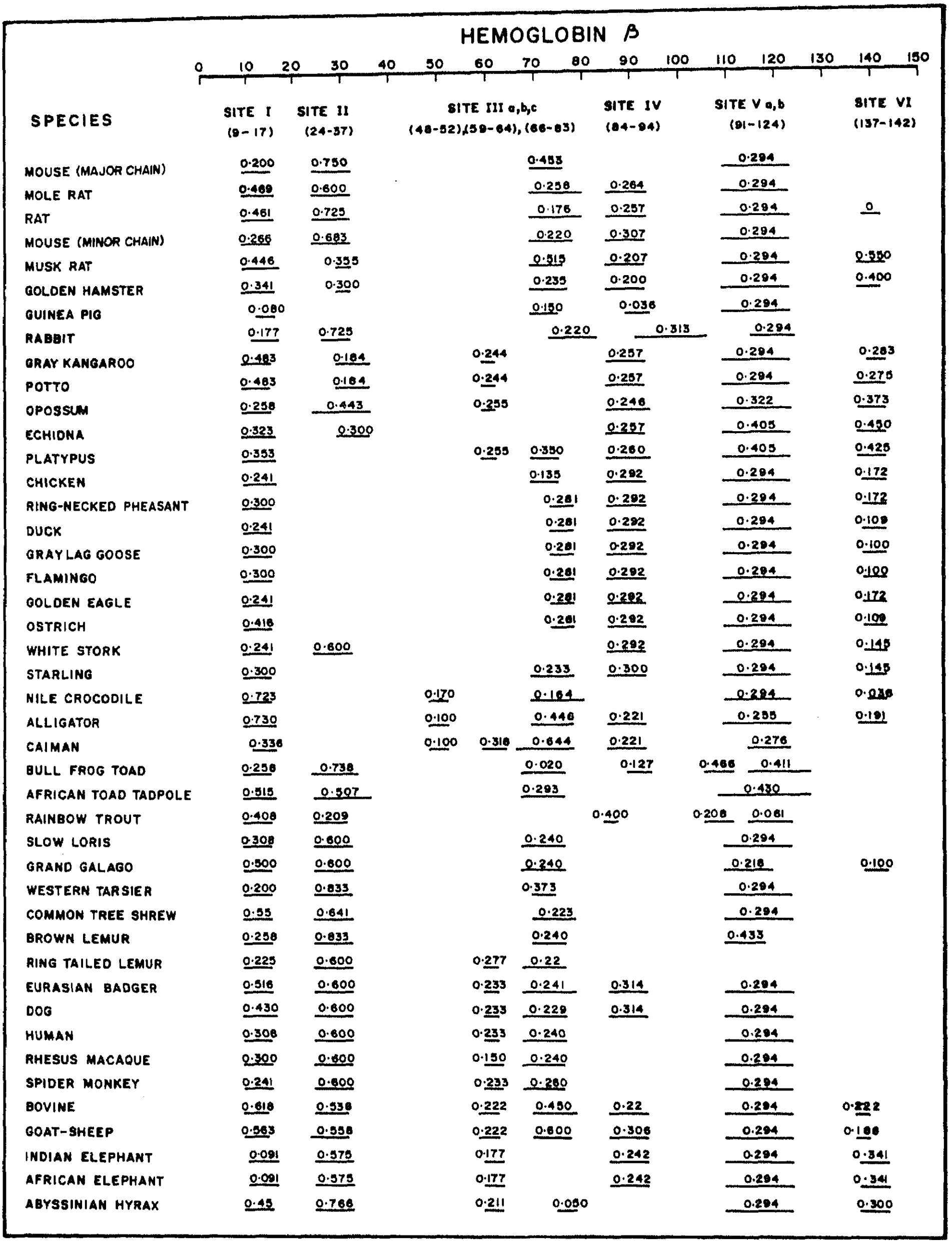
the range indicated by the sites picked up by using hydrophilicity values. Although sites I and IV picked up by the later approach are not found antigenic experimentally, reasonably good correlation was found in case of other sites. Several hemoglobins from various species included in the study are evolutionarily distant and have very little sequence homology. However, it can be seen from figures 3 and 4 that sites I, III and V in the case of α-chains of hemoglobins and sites I and V in the case of β-chains of hemoglobins have changed very little during the evolution. It is interesting to note that in the case of sites II and IV of α-chains of hemoglobins there is a significant variation in terms of either a splitting of a site, or a drastic change in the hydrophilicity values, or even a complete absence of any of these sites. In addition, site VI is missing in most of the α-chains of hemoglobins with very few exceptions. In the case of β-chains of hemoglobins there is a significant variation in sites II, III and VI, and a complete absence of site II in most of the birds and the reptiles analysed. These specific observations may have a bearing on the evolution of these species. Comparison of hydrophilicity data of hemoglobins with myoglobins from all the species shows that site VII present on myoglobin is completely deleted in case of α- and β-chains of hemoglobins.

These observations open up many questions in relation to their significance and suggest the need for more scrutiny of such data. It was thought that it would be possible to meaningfully interpret these results by comparing the data on myoglobins, α- and β-chains of hemoglobin from the same species. We could pick up 17 species where the sequences for all the 3 proteins were available from the data



HEMOGLOBIN α

SPECIES	SITE I (9–17)	SITE II a,b,c (19–42)	SITE III (57–79)	SITE IV (83–94)	SITE V a,b (105–127)	SITE VI a,b (137–142) (149–153)
YELLOW BABOON	1·000	0·072	0·520		0·263	
BROWN LEMUR	0·530	0·381	0·356		0·263	
SLOW LORIS	0·523	0·075	0·325		0·263	
TARSIER	0·523	0·050	0·406		0·263	
TREE SHREW	0·592		0·175		0·354	
MOUSE	0·823		0·356		0·263	
MUSK RAT	0·823		0·356		0·263	
GOLDEN HAMSTER	0·792		0·356		0·263	
GUINEA PIG	0·546	0·041	0·472		0·263	
RABBIT	0·507	0·011 0·227	0·461		0·263	
EUROPEAN HEDGHOG	0·575	0·245	0·356	0·110	0·263	
MUSK SHREW	0·530	0·108 0·281	0·325	0·253	0·263	
EUROPEAN MOLE	0·561	0·145 0·290	0·325		0·263	
DOG	0·569	0·075 0·390	0·325		0·233	
BADGER	0·500	0·327	0·356		0·233	
AFRICAN ELEPHANT	0·815	0·127	0·387		0·263	
EGYPTIAN FRUIT BAT	0·523	0·254	0·387		0·263	
ROCK HYRAX	0·569	0·058	0·080		0·263	0·390
HORSE	0·484		0·356		0·263	
TAPIR	0·530		0·241		0·233	
WHITE RHINOCEROS	0·538		0·356		0·263	
NINE BANDED ARMADILLO	0·508		0·325		0·263	
PIG	0·476		0·205		0·263	0·181
BOVINE	0·515		0·071	0·228	0·263	
GAYAL	0·515	0·027	0·071		0·263	
GRAY KANGAROO	0·541		0·150	0·225	0·263	
OPOSSUM	0·576		0·181		0·263	0·136
ECHIDNA	0·791	0·535	0·359		0·263	
PLATYPUS	0·830	0·514	0·392	0·300	0·263	
CHICKEN	0·469	0·533 0·277	0·180		0·263	
STARLING	0·625	0·464 0·277	0·247	0·218	0·327	
DUCKS	0·608	0·450 0·277	0·283		0·327	
GRAY LAG GOOSE	0·608	0·450 0·277	0·283		0·327	
HUMAN	0·523		0·356	0·04	0·263	
FLAMINGO	0·538	0·227	0·04		0·327	
GOLDEN EAGLE	0·515	0·414	0·340		0·327	
WHITE STORK	0·553	0·414 0·277	0·107 0·041		0·327	
RHEA	0·538	0·244 0·277	0·253	0·263	0·327	
NILE CROCODILE	0·875	0·377	0·116	0·010	0·133	
ALLIGATOR	0·850	0·442	0·240	0·010	0·133	
CAIMAN	1·150	0·377	0·126		0·122	
CHICKEN	0·733	0·388	0·511	0·100		0·136
RING NECKED PHEASANT	0·783	0·136 0·200	0·494	0·100		0·136
MUSCOVY DUCK	0·591	0·440	0·431	0·0818		0·263
PAINTED TURTLE	0·725	0·309	0·411	0·163		
SNAKE NECKED TURTLE	0·409	0·258	0·442			
ASPIC VIPER	1·15	0·464	0·357	0·209	0·418	0·050
BULL FROG TADPOLE	0·390			0·13	0·088	
AFRICAN CLAWED FROG α MAJOR	0·664	0·409	0·100	0·253	0·090 0·400	
ROUGHSKIN NEWT	0·735	0·428	0·023 0·077	0·009	0·425	0·05
AXOLOTL	0·978	0·400	0·536 0·077		0·325	
CARP	1·125	0·692	0·207	0·400	0·422	
DESERT SUCKER	1·269	0·692	0·207	0·318	0·500	
GOLD FISH	1·15	0·692	0·425	0·472	0·288	
RAINBOW TROUT	0·866	0·363 0·225	0·227		0·377	
SOUTH AMERICAN LUNGFISH	0·935	0·145	0·545		0·466	0·090 0·16



SPECIES	SITE I (9–17)	SITE II (24–37)	SITE III a,b,c (48–52),(59–64),(66–83)	SITE IV (84–94)	SITE V a,b (91–124)	SITE VI (137–142)
MOUSE (MAJOR CHAIN)	0·200	0·750	0·453		0·294	
MOLE RAT	0·469	0·600	0·258	0·264	0·294	
RAT	0·461	0·725	0·176	0·257	0·294	0
MOUSE (MINOR CHAIN)	0·266	0·683	0·220	0·307	0·294	
MUSK RAT	0·446	0·355	0·515	0·207	0·294	0·550
GOLDEN HAMSTER	0·341	0·300	0·235	0·200	0·294	0·400
GUINEA PIG	0·080		0·150	0·036	0·294	
RABBIT	0·177	0·725	0·220	0·313	0·294	
GRAY KANGAROO	0·483	0·184	0·244	0·257	0·294	0·283
POTTO	0·483	0·184	0·244	0·257	0·294	0·275
OPOSSUM	0·258	0·443	0·255	0·246	0·322	0·373
ECHIDNA	0·323	0·300		0·257	0·405	0·450
PLATYPUS	0·353		0·255 0·350	0·260	0·405	0·425
CHICKEN	0·241		0·135	0·292	0·294	0·172
RING-NECKED PHEASANT	0·300		0·281	0·292	0·294	0·172
DUCK	0·241		0·281	0·292	0·294	0·109
GRAY LAG GOOSE	0·300		0·281	0·292	0·294	0·100
FLAMINGO	0·300		0·281	0·292	0·294	0·100
GOLDEN EAGLE	0·241		0·281	0·292	0·294	0·172
OSTRICH	0·416		0·281	0·292	0·294	0·109
WHITE STORK	0·241	0·600		0·292	0·294	0·145
STARLING	0·300		0·233	0·300	0·294	0·145
NILE CROCODILE	0·723		0·170 0·164		0·294	0·036
ALLIGATOR	0·730		0·100 0·446	0·221	0·255	0·191
CAIMAN	0·336		0·100 0·318 0·644	0·221	0·276	
BULL FROG TOAD	0·256	0·738	0·020	0·127	0·466 0·411	
AFRICAN TOAD TADPOLE	0·515	0·507	0·293		0·430	
RAINBOW TROUT	0·408	0·209		0·400	0·208 0·061	
SLOW LORIS	0·308	0·600	0·240		0·294	
GRAND GALAGO	0·500	0·600	0·240		0·218	0·100
WESTERN TARSIER	0·200	0·833	0·373		0·294	
COMMON TREE SHREW	0·55	0·641	0·223		0·294	
BROWN LEMUR	0·258	0·833	0·240		0·433	
RING TAILED LEMUR	0·225	0·600	0·277 0·22			
EURASIAN BADGER	0·516	0·600	0·233 0·241	0·314	0·294	
DOG	0·430	0·600	0·233 0·229	0·314	0·294	
HUMAN	0·308	0·600	0·233 0·240		0·294	
RHESUS MACAQUE	0·300	0·600	0·150 0·240		0·294	
SPIDER MONKEY	0·241	0·600	0·233 0·260		0·294	
BOVINE	0·618	0·538	0·222 0·450	0·22	0·294	0·222
GOAT-SHEEP	0·563	0·558	0·222 0·600	0·306	0·294	0·188
INDIAN ELEPHANT	0·091	0·575	0·177	0·242	0·294	0·341
AFRICAN ELEPHANT	0·091	0·575	0·177	0·242	0·294	0·341
ABYSSINIAN HYRAX	0·45	0·766	0·211 0·050		0·294	0·300

Figure 4. Hydrophilic sites on *β*-chains of hemoglobin from various species (the average hydrophilicity values of individual sites are given along with the sites).

an appreciable variation in hydrophilicity value. There was also a merging of sites IV and V in the case of myoglobins from two species. In the case of *α*- and *β*-chains of hemoglobins the invariance in length of sites and their hydrophilicity values is maintained in only few sites (sites I and V). The variations, wherever they occurred, are reflected in terms of either splitting or shifting of the hydrophilic site, or change in the length of the site. In their studies on mammalian hemoglobins and

Table 2. Comparison of the sites on α-hemoglobin obtained by using average hydrophilicity values with those observed experimentally by earlier workers[b,c].

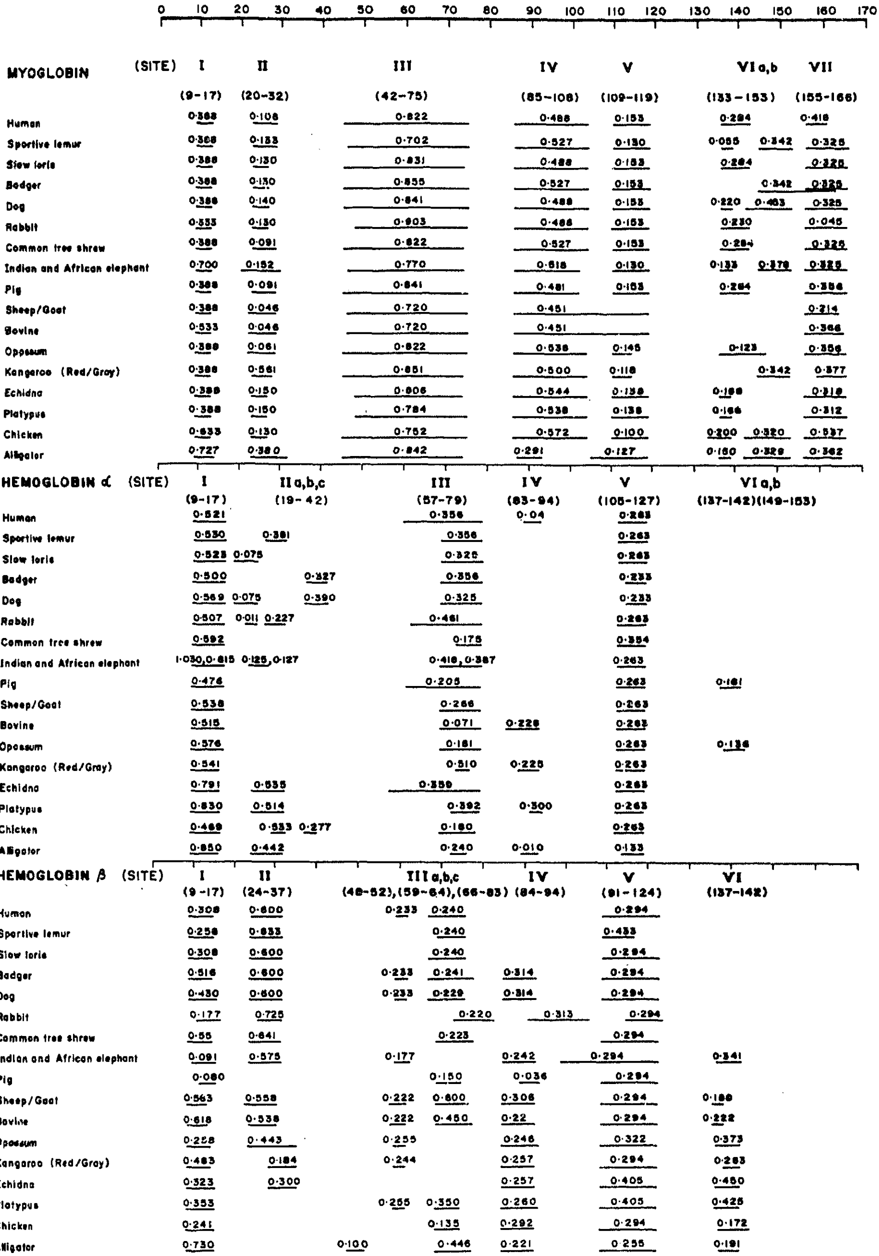
Site No.	Site based on hydrophilicity[a]	Experimentally observed sites			
		Human[b]	Rabbit[c]	Mouse[c]	Goat[c]
I	9– 17	—	—	—	—
IIa,b,c	19– 42	20– 37	21– 36	21– 36	24– 32
III	57– 79	53– 84	70– 83	70– 83	62– 75
IV	83– 94	—	—	—	—
Va,b	105–127	106–122	107–121	107–121	107–121
VIa	137–142	128–142	126–134	126–134	129–141
VIb	149–153	138–162	149–161	149–161	146–154

[a]Sites are depicted by the range covered by various species shown in figure 3.
[b]Kazim and Atassi (1980).
[c]Kazim and Atassi (1982).

myoglobins based on the nucleotide replacements, Barnabas *et al.* (1978) have suggested that primate myoglobins evolve at a slower rate than primate hemoglobins. Our observations are also suggestive of the fact that hemoglobins are evolving faster than myoglobins. Tetrameric hemoglobin is known to carry out variety of functions not observed with myoglobin. Relatively faster evolution of hemoglobins may be the manifestation of the much diversified functional demands placed on them.

Comparison of hydrophilicity values averaged over all the species for the 3 proteins are given in figure 6. This figure allows one to see the differences in the hydrophilic character of various sites on different proteins irrespective of species being studied. It can be seen from figure 6 that hydrophilic characteristics of sites I to IV on myoglobins are drastically different from the other proteins while they are more or less same in case of sites V and VI. Site III on myoglobins has highest hydrophilic character amongst all the sites, while site II is appreciably weaker in this respect. In keeping with the single crystal structures obtained by X-ray diffraction method for deoxymyoglobin and hemoglobin (Dickerson and Geis, 1983), almost all the residues which we have predicted as antigenic are found to be on the surface of the molecules. It may be mentioned here that we have determined antigenic determinants on α- and β-chains independently in their monomeric forms. However, when hemoglobin molecule is formed, a few residues which are on the surface of the individual α- and β-chains do not remain on the surface but fall in the interior of the molecule. In the case of hemoglobins site I is more hydrophilic in α-chains as compared with β-chains while reverse is the case with site II, thus explaining the maintenance of delicate balance in hydrophilic character of the protein as mentioned earlier. In general it can be said that in all species hydrophilic regions show preference for the N-terminal half of the protein chain making these regions of the chain more exposed to the environment. The data further suggest that in case of hemoglobins the hydrophilic regions are much more flexible and have evolved due to the strong interactions with the environment. It is known that hemoglobins have evolved from myoglobins, leading to α- and β-forms most probably through the mechanism of gene duplication.

The above observations indicate that in case of related proteins the changes in



MYOGLOBIN (SITE)	I (9-17)	II (20-32)	III (42-75)	IV (85-108)	V (109-119)	VI a,b (133-153)	VII (155-166)
Human	0·388	0·108	0·822	0·488	0·153	0·284	0·418
Sportive lemur	0·388	0·133	0·702	0·527	0·130	0·055 0·342	0·325
Slow loris	0·388	0·130	0·831	0·488	0·153	0·284	0·325
Badger	0·388	0·130	0·855	0·527	0·153	0·342	0·325
Dog	0·388	0·140	0·841	0·488	0·153	0·220 0·463	0·325
Rabbit	0·333	0·130	0·903	0·488	0·153	0·230	0·045
Common tree shrew	0·388	0·091	0·822	0·527	0·153	0·284	0·325
Indian and African elephant	0·700	0·152	0·770	0·518	0·130	0·133 0·378	0·325
Pig	0·388	0·091	0·841	0·481	0·153	0·284	0·384
Sheep/Goat	0·388	0·046	0·720	0·451			0·214
Bovine	0·533	0·046	0·720	0·451			0·366
Opossum	0·388	0·061	0·822	0·536	0·145	0·123	0·356
Kangaroo (Red/Gray)	0·388	0·561	0·851	0·500	0·118	0·342	0·377
Echidna	0·388	0·150	0·806	0·544	0·138	0·188	0·318
Platypus	0·388	0·150	0·784	0·538	0·138	0·166	0·312
Chicken	0·633	0·130	0·752	0·572	0·100	0·200 0·320	0·537
Alligator	0·727	0·380	0·842	0·291	0·127	0·160 0·329	0·362

HEMOGLOBIN α (SITE)	I (9-17)	II a,b,c (19-42)	III (57-79)	IV (83-94)	V (105-127)	VI a,b (137-142)(149-153)
Human	0·521		0·356	0·04	0·263	
Sportive lemur	0·530	0·381	0·356		0·263	
Slow loris	0·523	0·075	0·325		0·263	
Badger	0·500	0·327	0·356		0·233	
Dog	0·569	0·075 0·390	0·325		0·233	
Rabbit	0·507	0·011 0·227	0·461		0·263	
Common tree shrew	0·592		0·175		0·354	
Indian and African elephant	1·030, 0·815	0·125, 0·127	0·416, 0·387		0·263	
Pig	0·476		0·205		0·263	0·161
Sheep/Goat	0·538		0·266		0·263	
Bovine	0·515		0·071	0·228	0·263	
Opossum	0·576		0·181		0·263	0·136
Kangaroo (Red/Gray)	0·541		0·510	0·225	0·263	
Echidna	0·791	0·535	0·359		0·263	
Platypus	0·830	0·514	0·392	0·300	0·263	
Chicken	0·469	0·533 0·277	0·180		0·263	
Alligator	0·850	0·442	0·240	0·010	0·133	

HEMOGLOBIN β (SITE)	I (9-17)	II (24-37)	III a,b,c (48-52),(59-64),(66-83)	IV (84-94)	V (91-124)	VI (137-142)
Human	0·308	0·600	0·233 0·240		0·294	
Sportive lemur	0·258	0·633	0·240		0·433	
Slow loris	0·308	0·600	0·240		0·294	
Badger	0·516	0·600	0·233 0·241	0·314	0·294	
Dog	0·430	0·600	0·233 0·229	0·314	0·294	
Rabbit	0·177	0·725	0·220	0·313	0·294	
Common tree shrew	0·55	0·641	0·223		0·294	
Indian and African elephant	0·091	0·575	0·177	0·242	0·294	0·341
Pig	0·080		0·150	0·036	0·294	
Sheep/Goat	0·563	0·558	0·222 0·600	0·306	0·294	0·188
Bovine	0·618	0·538	0·222 0·450	0·22	0·294	0·222
Opossum	0·258	0·443	0·255	0·246	0·322	0·373
Kangaroo (Red/Gray)	0·483	0·184	0·244	0·257	0·294	0·263
Echidna	0·323	0·300		0·257	0·405	0·450
Platypus	0·353		0·255 0·350	0·260	0·405	0·425
Chicken	0·241		0·135	0·292	0·294	0·172
Alligator	0·730		0·100 0·446	0·221	0·255	0·191

Figure 5. Comparative data on hydrophilic sites on myoglobins, α- and β-chains of hemoglobins from various species.

characteristics of antigenic sites are maintained at the minimal level and, efore, the hydrophilic sites on a group of related proteins could be used to tune lata on the antigenic determinants obtained experimentally for a single protein.

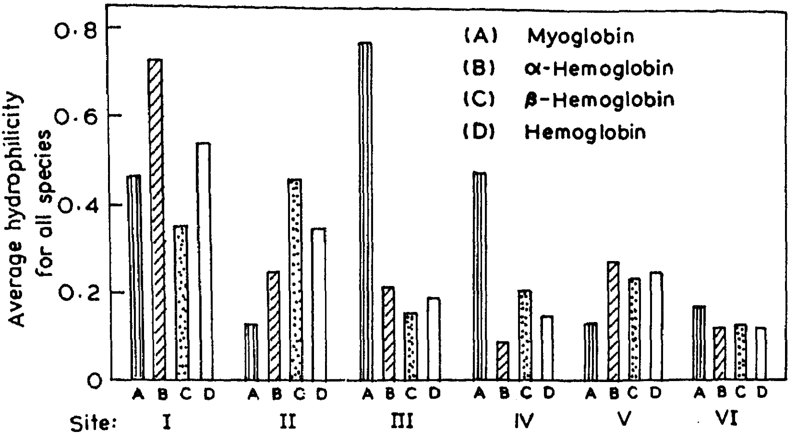


Figure 6. Hydrophilicity for various sites for 3 proteins averaged over all the species analysed.

The results also support the view that hemoglobins which perform a variety of functions are evolving faster than myoglobins.

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Heat shock response in ovarian nurse cells of *Anopheles stephensi*

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Abstract. Transcriptional and translational changes following temperature shock at 37, 39 or 41°C to ovarian cells of *Anopheles stephensi* were studied. Temperature shock at 39°C induced 6 puffs on polytene chromosomes in the nurse cells as revealed by [^{3}H] uridine incorporation studies. Only the 2R-19B puff was induced at 37°C and was found to be a major temperature shock locus remaining most active at all the 3 temperatures tested. Other temperature shock loci were activated only at 39°C. There was progressive inhibition of general chromosomal transcription with the rise of temperature. Transcription was drastically inhibited at 41°C but all the temperature shock loci still remained relatively active. Examination of [^{35}S]methionine labelled newly synthesized ovarian proteins using sodium dodecyl sulphate-polyacrylamide slab gels revealed that all the heat shock polypeptides except the HSP 70 were synthesized in ovarian cells even at control temperature (29°C). Temperature shock induced the synthesis of HSP 70 and elevated the levels of other heat shock polypeptides (82, 30, 29, 23 and 17 KD). Present results suggest that the threshold level for induction of a complete heat shock response in mosquitos is higher (39°C) than the other dipteran insects studied and that a 41°C treatment is not lethal as in the case of *Drosophila*, *Chironomus* etc. These features reflect the adaptations of mosquitos to tropical climate and their dietary habit of warm blood meal.

Keywords. Temperature shock; mosquito; heat shock polypeptides; polytene chromosomes; puffs; thermotolerance.

Introduction

Studies on heat shock response in a tropical chironomid, *Chironomus striatipennis*, revealed certain intriguing features, different from the typical *Drosophila*-like response (Nath and Lakhotia, 1989). The threshold level for the induction of heat shock loci was higher (39°C) in *C. striatipennis* than in *Drosophila* and other *Chironomus* species living in temperate zones. These and other features of the heat shock response in *C. striatipennis* (Nath and Lakhotia, 1989) suggested an important role of the heat shock response in tropical adaptations of these insects. These results provided an impetus to examine the heat shock response in other tropical dipteran insects. Mosquitos are interesting in this context because not only their population density is found to be higher during summer months (Pal, 1974) but also they are adapted to warm blood meals which in other dipterans like *Drosophila* may be warm enough to induce a heat shock response.

Anopheles stephensi is widespread in its distribution and is an important malaria vector in the tropical areas (see Rao, 1981). Polytene chromosomes are found in larval salivary glands of *A. stephensi*, transiently appearing during mid to late 4th the gonotrophic (post-blood meal) cycle. The ovarian nurse cell polytene chromosomes have been a favourable material for studies because of the ease with which preparations can be made and also because the chromosomes are of superior morphology compared to those in larval salivary glands (Redfern, 1981; Macgregor and Verley, 1983). The present study explores the basic heat shock response at levels of transcription and translation in the ovarian nurse cells of *A. stephensi.* The results were briefly presented earlier (Nath, 1987).

Materials and methods

Animal culture

Adults and larvae of *A. stephensi* (Delhi strain: stock obtained from the Malaria Research Centre, Delhi) were reared using the standard methods in an insectary maintained at 29 ± 1°C with a relative humidity of about 80%. Adults were fed on water soaked raisins.

To initiate ovarian development, 4–5 day old females were allowed a blood meal on rabbit. After the blood meal (24–30 h), ovaries were pulled out from the semi-gravid females and subjected to heat shock at different temperature in the inorganic salt constituents of Poels' tissue culture medium (Lakhotia and Mukherjee, 1980). Ovaries were either used for [^{3}H]uridine labelling to study transcriptional pattern or for [^{35}S] methionine labelling to study protein synthesis pattern after heat shock.

RNA synthesis after heat shock

The excised ovaries were heat shocked at 37, 39 or 41°C (treated) or were kept at 29°C (control) for 60 min. The treated and control ovaries were labelled with [^{3}H] uridine (activity 40 μCi/ml; specific activity 75 Ci/mM; Amersham, UK) for 10 min at respective temperatures. After labelling, ovaries were fixed in 1:3 aceto-methanol for 1 min and stained in 1% aceto-orcein. Finally the ovaries were dissociated in 50% propionic acid using fine needles to separate the ovarioles. A gentle tapping of the coverglass completed the squashing.

After squashing, the slides were either frozen on dry ice or in liquid nitrogen and coverslips were removed in the usual manner. The preparations were processed for autoradiography as described by Nath and Lakhotia (1989). Identification of heat shock loci was carried out using the polytene chromosome maps of Coluzzi *et al.* (1970).

Protein synthesis after heat shock

Excised ovaries were heat shocked at 37, 39 or 41°C or were incubated at 29°C (control) for 60 min and then labelled with [^{35}S]methionine (activity 100 μCi/ml; specific activity >800 Ci/mM; Amersham, UK). Following labelling, ovaries were washed in radio-isotope free medium and transferred to sodium dodecyl sulphate (SDS)-sample buffer (Laemmli, 1970) containing 2 mM PMSF. Samples were then heated in a boiling water bath for 5 min after mild homogenization and were immediately cooled on ice. The samples were kept frozen at −70°C until ectrophoresis. Electrophoresis followed by fluorography was carried out on SDS- lyacrylamide (10–15% gradient) slabs as described earlier (Singh and Lakhotia, 88).

esults

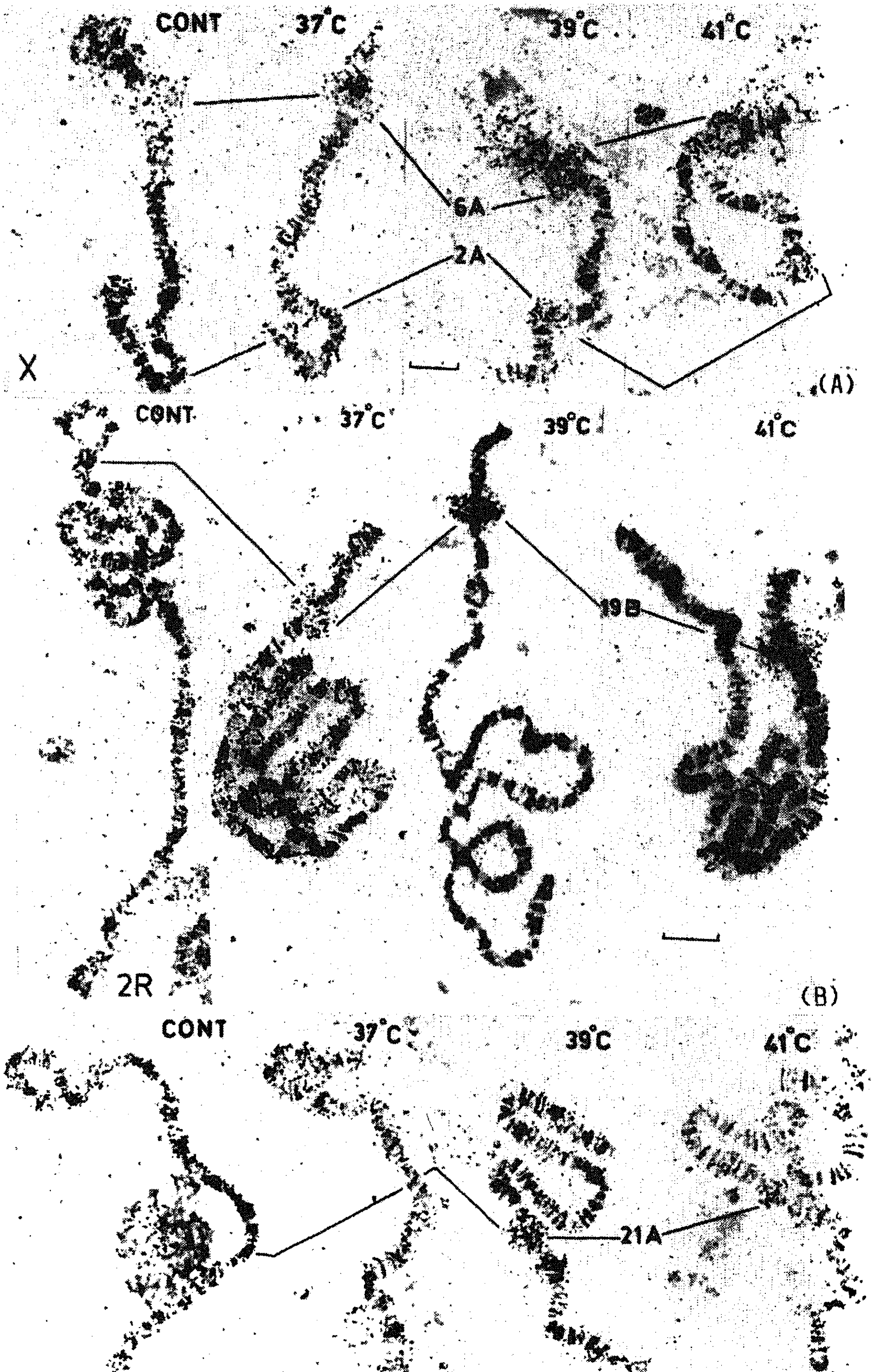
duction of puffs and [^{3}H]uridine incorporation after heat shock at different mperatures in A. stephensi

fect of heat shock on nurse cell polytene chromosomes was studied at 3 different mperatures *viz.*, 37, 39 and 41°C. The autoradiographic analysis of [^{3}H]uridine corporation revealed that heat shock caused significant changes in transcriptional tivity with the activation of a number of loci on different arms of polytene chromo- mes (figure 1). Transcriptional activity of these temperature shock (TS) loci was ored on the basis of grain counts (figure 2A). A chromosome segment, 28A to 28E 2L was scored to estimate the general chromosomal transcription. This chromo- mal segment was chosen because it did not exhibit any developmentally active or S induced puff. The relative transcriptional activity (figure 2B) of a TS locus was alysed as a ratio of the grain counts on the TS puff with respect to that on the -28A to 28E chromosomal segment of the same nucleus. A total of 6 heat shock ci were identified on the basis of their [^{3}H]uridine labelling at different mperatures. These were located as follows: two on X-chromosome (X-2A and X-); one each on right and left arms of chromosome 2 (2R-19B and 2L-21A) and o on the left arm of chromosome 3 (3L-38B and 3L-40E). Right arm of chromo- me 3 did not show any heat shock induced locus (see figure 1). With increasing at shock temperature from 37–41°C there was a progressive inhibition of general romosomal transcription (see figure 2A).

The most obvious change after a 37°C shock was induction of the 19B locus on ht arm of chromosome 2. Mean grain counts on other TS loci were comparable those in control (figure 2A). Since the gereral chromosomal labelling was partially hibited, the relative transcriptional activity of each TS locus showed a higher level 37°C than at 29°C (figure 2B).

At 39°C, all the other heat shock loci, in addition to the 19B locus, displayed nspicuous incorporation of [^{3}H]uridine (figure 1). In X chromosome, 6A was anscriptionally more active than 2A at 39°C (figure 1A). Left arm of chromosome showed a minor TS puff at 21A (figure 1C) with its transcriptional activity being s than the other loci induced at 39°C. Two other TS loci were induced at 39°C left arm of chromosome 3, *viz.*, 38B and 40E (figure 1E). Among all the TS loci, e 19B on 2R showed maximal induction at 39°C.

At 41°C, transcriptional activity of all the TS loci was drastically reduced (figure) with mean grain counts on the different TS puffs generally less than those on rresponding controls. However, when relative transcriptional activity was ana- sed, all the TS loci were found to show higher relative activity levels than the rresponding control values (figure 2B) since the general chromosomal transcrip- on was more severely inhibited at 41°C (see figure 2A). A comparison of overall ttern of heat shock response showed that 19B was the major TS puff remaining tive at all the temperatures tested and of all the TS loci it showed maximal belling.


CONT
37°C
39°C
41°C
6A
2A
X
(A)
CONT
37°C
39°C
41°C
19B
2R
(B)
CONT
37°C
39°C
41°C
21A

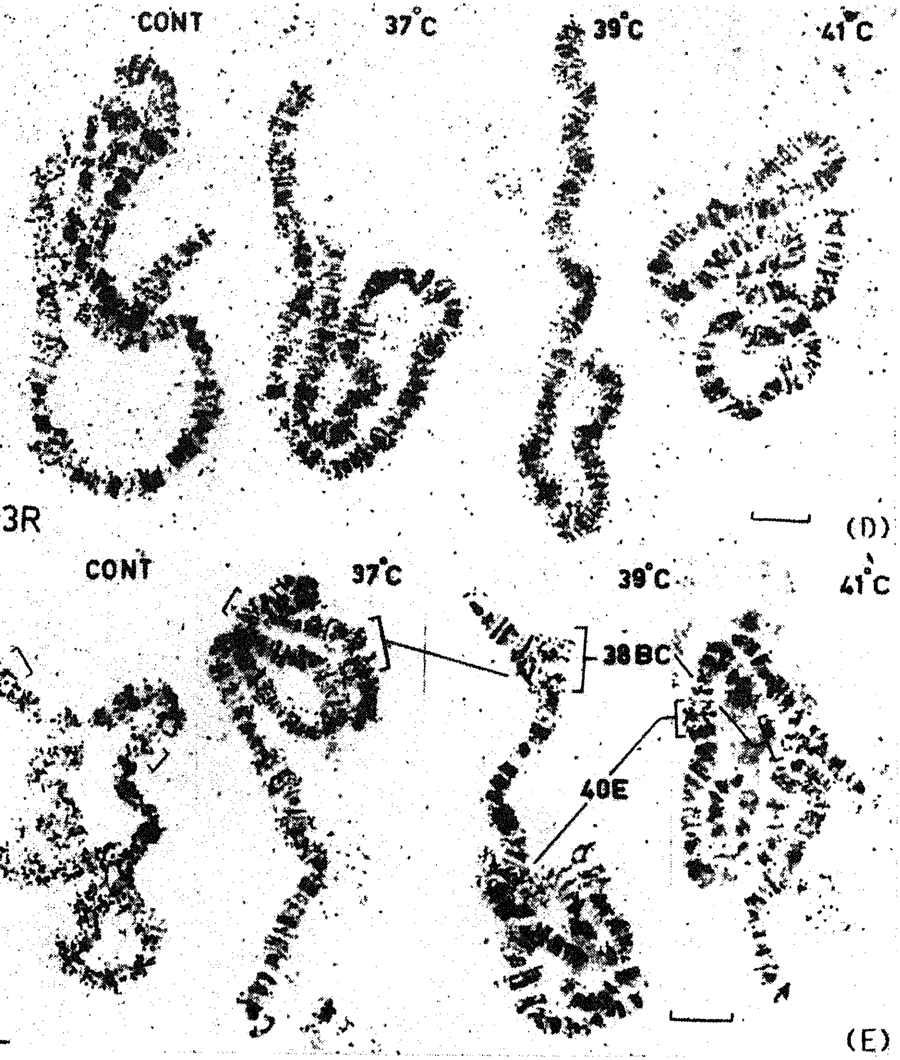


Figure 1. [^{3}H]Uridine labelled polytene chromosomes showing heat shock loci on X-chromosome (A), right arm of chromosome 2 (B), left arm of chromosome 2 (C), right arm of chromosome 3 (D), and left arm of chromosome 3 (E) from control (29°C) and heat shocked (TS 37°C, TS 39°C, TS 41°C) ovarian nurse cell polytene nuclei of *A. stephensi*. The bars represent 10 μm.

ect of heat shock on ovarian protein synthesis

at shock to ovaries of *A. stephensi* did not result in synthesis of any novel ypeptide, except a 70 KD species. However, synthesis of several discrete species polypeptides, already synthesized in control ovaries, was elevated after heat ck. The estimated molecular weights of these polypeptides were 82, 30, 29, 23 l 17 KD (see figure 3). Based on the criterion that the synthesis of these ypeptides was stimulated after heat shock, they are referred to as heat shock ypeptides (HSPs) (Schlesinger, 1986).

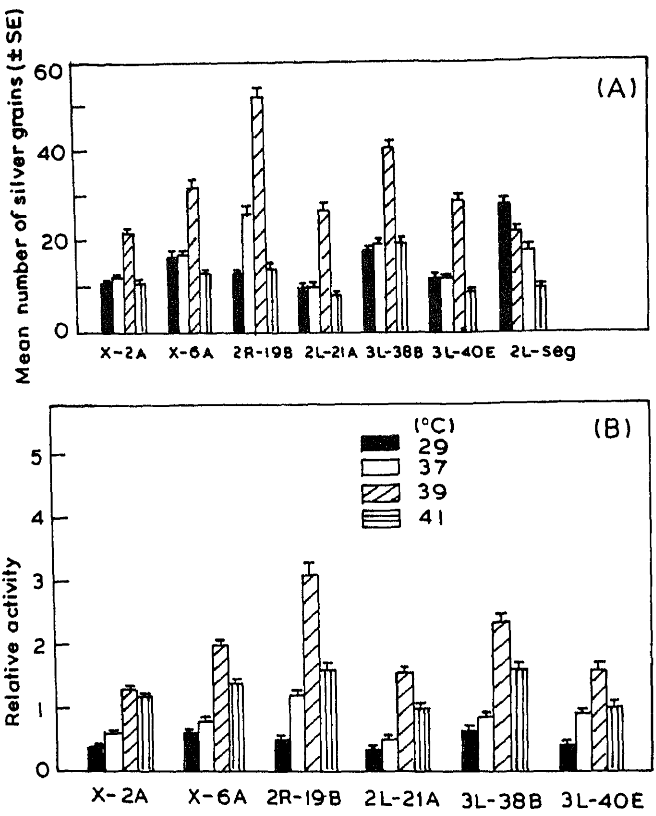


Figure 2. [^{3}H] Uridine incorporation (ordinate) on different heat shock loci and th segment of 2L (abscissa) at different temperatures. The [^{3}H] uridine uptake is shown i terms of mean numbers of silver grains ± SE in (A) and as the relative transcriptiona activity of different heat shock loci in (B). For each data point 20–25 nuclei were scored.

Upon heat shock at 37°C, the synthesis of HSP 82, HSP 70, HSP 30 and HSP 2 was elevated to higher levels than in control ovaries and this persisted at 39°C a well. HSP 23 and HSP 17 were abundantly synthesized after heat shock at 37 an 39°C, although their levels seemed to be slightly lower than in control ovarie (figure 3). However, since the general protein synthesis was more inhibited at 37 an 39°C, HSP 23 and 17 also appeared prominent in fluorograms of 37 and 39°C lane A progressive inhibition of general protein synthesis was noted with increasing hea shock temperature: degree of inhibition was not much pronounced at 37°C but wa severe at 41°C. After a 41°C shock, HSP 82, 70 and 17 were still faintly detecte Longer fluorographic exposure of the 41°C lane (see figure 3, lane D′ and confirmed that all the HSPs as well as other polypeptides were still being synthesize after the 41°C heat shock.

Discussion

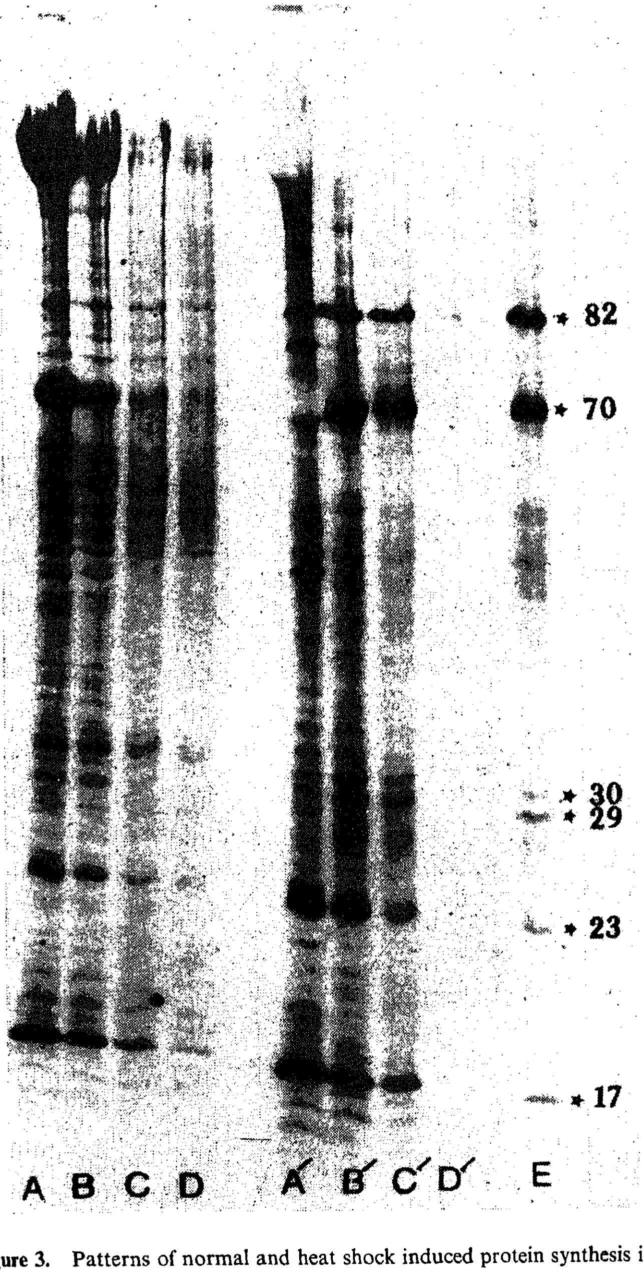


Figure 3. Patterns of normal and heat shock induced protein synthesis in ovaries of adult *Anopheles*: Lanes A–D show the CBB staining patterns in 29°C control (lane A) and in heat shocked (37°C, lane B; 39°C, lane C and 41°C, lane D) ovaries. The corresponding fluorograms are shown in lanes A′–D′ respectively after a 6 day exposure. Lane E shows a 15 day exposure of the 41°C-lane. Molecular weights (in KD) of the HSPs are marked.

Although Berger *et al.* (1985) have reported the protein synthesis pattern in heat shocked *Aedes* cell line, detailed studies on heat shock response in mosquitos at the level of transcription and translation are lacking. In *Anopheles*, heat shock response, although analogous to other dipteran insects has some distinctive features. In *Drosophila* and in a local species of *Chironomus* (Nath and Lakhotia, 1989), the heat shock genes begin to show induction at 33°C. In contrast, in the case of *A. stephensi*, even at 37°C, only one of the TS loci showed elevated level of [^{3}H]uridine uptake; full complement of TS puffs was induced only at 39°C. The reason for only a marginal induction in *Anopheles*, even at 37°C can be attributed to its higher optimal growth temperature (29°C) than that of *Drosophila* or *Chironomus* (20–25°C) and other biological adaptations.

The two heat shock loci at 6A and 2A are notable in *Anopheles* due to their X-linkage. Since mosquitos have an XX/XY sex-chromosome system (King, 1975), X-linkage of these heat shock genes becomes of interest from the view point of dosage compensation (Muller, 1932). In the genus *Drosophila*, all heat shock genes are presumed to be originally autosomal and only in some species, a few of the heat shock genes become X-linked due to autosome-X translocation during evolution (Sturtevant and Tan, 1937; Pierce and Lucchesi, 1980). Interestingly, these neo X-linked heat shock genes have evolved a dosage compensating regulatory system (Lucchesi, 1978; Pierce and Lucchesi, 1980; Strobel *et al.*, 1978). In this context, the evolutionary origin of the X-linked heat shock genes in *Anopheles* and their dosage compensation become interesting issues for further study.

At the level of protein synthesis, ovarian nurse cells exhibited enhanced synthesis of a set of polypeptides at elevated temperatures. Among the temperature induced ovarian HSPs, 82 and 70 KD were the most prominently labelled species. Except for the HSP 70, all other HSPs were also synthesized in ovaries of *A. stephensi* even at control temperature (29°C). In this context it may be noted that the constitutive synthesis of the various HSPs in ovarian cells of mosquito is unlikely to be due to heat shock that may be caused by the previous warm blood-meal since the time interval between the warm blood-meal and examination of protein synthesis in ovarian cells was long enough (24–30 h) to let the cells recover from the heat shock, if any. There is growing evidence of the presence of HSPs under non-heat shock conditions in specific developmental stages/cell types in different organisms (Lindquist, 1986; Bienz and Pelham, 1987). HSPs appear to be normal component of larval imaginal discs (Cheney and Shearn, 1983) and are also found in normal *Drosophila* embryos (Graziosi *et al.*, 1980; Bergh and Arking, 1984; Zimmerman *et al.*, 1983; Mason *et al.*, 1984). Heat shock mRNAs are also reported to be constitutively present in *Xenopus* oocytes (Bienz, 1985; Browder *et al.*, 1987). In a tropical midge *C. striatipennis* most HSPs were constitutively synthesized in ovarian cells (Nath and Lakhotia, 1989). Thus a constitutive synthesis of HSPs in ovarian cells of mosquitos may reflect a common feature seen in a wide variety of organisms. In this context, the total absence of synthesis of HSP 70 in control ovaries of *A. stephensi* is rather surprising, since HSP 70 is the most conserved and most abundant of all heat shock proteins (Schlesinger *et al.*, 1982; Schlesinger, 1986; Nover 1984; Lindquist 1986). CBB staining, however, revealed a prominently stained band in the 70 KD range in control ovaries of *A. stephensi* (see figure 3, lane A) and if this represents HSP 70, the absence of the HSP 70 synthesis in these ovaries may be understood in terms of autoregulation such that once a threshold level is attained, further synthesis is inhibited (DiDomenico *et al.*, 1982). The presence of polypeptides corresponding in molecular weight to HSP 70 in control ovaries also raises the possibility that some of them might represent HSC 70 (cognates of HSP 70), which are known in *Drosophila* as well as yeast to be essential for growth at any temperature (Craig *et al.*, 1982; Craig and Jacobsen, 1984, 1985).

Present results showed that in *A. stephensi* ovaries, synthesis of HSP 70 alone was newly induced at 37°C while at the transcriptional level, the 19B puff on 2R alone was induced by the 37°C shock. In view of this, it is tempting to speculate that the 2R-19B locus may code for HSP 70. This correlation is further supported by the fact that the 2R-19B locus is the most active heat shock locus as would be expected for the most abundantly synthesized HSP 70. A final identification of the different HSP loci, however can only be made after further molecular studies.

A significant observation in this study was the persistence of general transcriptional and translational activities at 37°C or even at 39°C in *A. stephensi.* This contrasts with the near lethal effect of 39°C heat shock in *Drosophila.* Moreover the present results showed that *A. stephensi* cells continued some transcriptional and translational activities at 41°C, a temperature at which even cells of a tropical species of *Chironomus* and *C. striatipennis* were found (Nath and Lakhotia, 1989) to have stopped most of their synthetic activities. This shows that in the case of mosquito, the threshold level for sensing heat shock is set to a higher temperature and their cellular activities are adapted to continue functioning even at relatively warmer temperatures. A similar finding was reported by Berger *et al.* (1985) for cultured cells of another mosquito *Aedes albopictus.* While tropical adaptation by itself would set the heat shock threshold at a higher level, the dietary habit of blood meal of warm-blooded animals would also require that mosquitos do not suffer the heat stress at 37°C. The constitutive synthesis of HSPs in ovarian tissue of mosquito as found in this study may be one of the biological adaptation of these insects to tropical climates. Further studies will show if other tissues of mosquito also synthesize HSPs constitutively.

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Qualitative and quantitative variation of tRNA in certain invertebrates†

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Abstract. Total tRNA was isolated, purified and quantitated from earthworm, cockroach, fresh water mussel and rat liver. The total tRNA content of invertebrates was found to be much lower than that of rat liver. When checked for aminoacylation capacity with homologous and heterologous enzymes and algal protein hydrolysate, the tRNA preparation from rat liver and fresh water mussel, a mollusc, were found to be active. On the other hand, the tRNAs from earthworm, an annelid, and cockroach, an arthropod, were completely inactive with the homologous enzymes but showed partial activity with heterologous enzymes. Similar results were obtained with individual amino acids also. The low activity or inactivity of earthworm and cockroach tRNAs appears to be due to certain endogenous aminoacylation inhibitors.

Keywords. Transfer RNA; aminoacyl-tRNA synthetase; aminoacylation; earthworm; cockroach; fresh water mussel.

Introduction

Transfer RNAs play a central role in protein biosynthesis and they interact with a number of proteins and nucleic acids to carry out this function with fidelity (Rich and Schimmel, 1977). The central role of tRNA in translation of genetic code makes it likely that changes in the abundance and specificity of individual tRNAs may play an important role in metabolic regulation, cellular differentiation and neoplasia. Changes in tRNA population have been shown during growth (Chuang and Doi, 1972; Heyman *et al.*, 1973), viral infection (Kano-Sueoka and Sueoka, 1968; Natale and Eilat, 1976), differentiation and development (Yang and Comb, 1968; Zeikus *et al.*, 1967), ageing (Strehler *et al.*, 1969; Mays *et al.*, 1978) and neoplasia (Taylor *et al.*, 1967; Murphy and Cooper, 1982). In addition, tRNA molecule being an essential component of all biological systems, is an interesting subject for the study of evolution (Eigen and Winkler, 1981).

Information is available on the primary structure of tRNAs from prokaryotes, eukaryotes and organelles (Sprinzl *et al.*, 1985). A systematic analysis of the available data shows that tRNAs are much more conserved during evolution compared to other RNAs (Holmquist *et al.*, 1973; Rodriguez–Vargar *et al.*, 1984). However in the literature there is only limited information available in the area of invertebrates and the present study represents an initial attempt in this direction.

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Abbreviations used: BSA, Borine serum albumin; DTT, dithiothreitol; TCA, trichloroacetic acid.

Materials and methods

Animals

Earthworm (*Megascolex* sp), cockroach (*Periplaneta americana*) and fresh water mussel (*Lamellidens marginalis*) were purchased from a local supplier and sacrificed on the same day. Care was taken to collect the animals from the same location to minimize environmental variations. Earthworms ranging in size from 15–30 cm were pooled and used. In the case of cockroaches, adult species after removing the wings were used. As for the fresh water mussel (unio), the whole animal devoid of shell was used. The rats used as controls in various experiments were 30-week old female albino rats of Wistar strain bread in the departmental animal house.

Chemicals

DEAE-Cellulose (capacity 0·9 meq/g), Sephadex G-100 (40–120 μ), *Escherichia coli* W tRNA, 2-mercaptoethanol, ATP, CTP, calf thymus DNA, yeast total RNA, bovine serum albumin (BSA), dithiothreitol (DTT), PPO and POPOP were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. [^{14}C] Chlorella protein hydrolysate (42 mCi/m atom C) and [^{14}C] amino acids of high specific activities were from Bhaba Atomic Research Centre, Bombay. Filter paper discs of 2·5 cm diameter were cut out from Whatman 1 filter paper sheets (Whatman, England). All other reagents used were of analytical grade.

Extraction and purification of total tRNA

Transfer RNA was extracted from different animals according to the method of Dure (1973) with modifications as suggested by Manjula and Mayasundari (1981). All steps were carried out at 0–4°C unless and otherwise specified. The animals were minced and suspended in 0·01 M Tris-HCl, pH 7·5 containing 0·01 M KCl, 0·01 M $MgCl_2$, 0·001 M EDTA, 0·001 M 2-mercaptoethanol and 1% sodium deoxycholate (or 0·5% sodium dodecyl sulphate in case of earthworm and cockroach). Two ml buffer per gram wet weight of tissue in the case of rat liver and unio and 6 ml buffer in the case of earthworm and cockroach were used. The suspension was homogenized in a Waring blender for 5 min at low speed. The homogenate was centrifuged at 10,000 *g* for 20 min and the pellet was re-extracted. The pooled supernatant was extracted thrice with equal volume of buffer-saturated phenol for 30 min. The final aqueous layer was made 3% with potassium acetate, pH 5 and precipitated with 2·5 volumes of ice-cold ethanol. The precipitate was recovered by centrifugation at 10,000 *g* for 10 min and dissolved in a minimum volume of 0·01 M Tris-HCl, pH 7·5, 0·01 M $MgCl_2$ and 0·25 M NaCl. The sample was loaded onto a DEAE-cellulose column [50 A_{260} units/ml ion exchanger] pre-equilibrated with the same buffer at room temperature. Transfer RNA was eluted from the column using 1 M NaCl in the same buffer and fractions having A_{260} readings above 0·2 were pooled and precipitated with ice-cold ethanol. The pooled tRNA sample was applied to a Sephadex G-100 column at room temperature, previously equilibrated with 0·01 M Tris-HCl, pH 7·5, 0·4 M NaCl and 0·01 M $MgCl_2$

d eluted with the same buffer. The fractions eluting in the range of standard *coli* tRNA were pooled and used as purified tRNA. The amount of tRNA present s estimated on the basis of optical density.

Twenty A_{260} units were taken as equivalent to 1 mg of tRNA. In large scale paration the purified tRNA was precipitated with 2·5 volumes of ice-cold anol. Deacylation was carried out according to Dure (1973). The tRNA cipitate was dissolved in 1·8 M Tris-HCl, pH 8 and incubated at 37°C for 1 h d recovered by precipitation with 2·5 volumes of ice-cold ethanol, washed with 75 d 80% ethanol, ether, dried and stored at −20°C.

DNA and RNA concentrations of homogenate were estimated according to the thod of Schneider (1957) using calf thymus DNA and yeast total RNA standards. otein was estimated according to Lowry *et al.* (1951) using crystalline BSA as ndard.

paration of aminoacyl-tRNA synthetase

cal aminoacyl-tRNA synthetases were prepared from whole animals in the case of ertebrates, and from the liver in the case of rat, according to the method of Yang d Novelli (1971) with minor modifications. All operations were carried out at °C. The tissue was homogenized in a buffer containing 0·01 M Tris-HCl, pH 7·5, 05 M magnesium acetate, 0·01 M KCl, 0·005 M EDTA and 0·01 M 2-mercapto-anol. Three ml of buffer were used per gram wet weight of tissue. The mogenate was centrifuged at 14,000 *g* for 20 min. The supernatant was freed of osomes by further centrifugation at 150,000 *g* for 3 h in MSE-75 ultracentrifuge. e endogenous tRNA and amino acids present in the 150,000 *g* supernatant were noved by chromotography on DEAE-cellulose (2 mg protein/ml ion exchanger) ich was equilibrated with 0·01 M phosphate buffer, pH 7·5, 0·001 M $MgCl_2$, 02 M DTT and 0·005 M KCl. The column was washed with the same buffer and aminoacyl-tRNA synthetases were eluted with the same buffer containing 0·3 M Cl. Fractions having A_{280} values above 0·5 were pooled and used as the enzyme. e enzyme was dialysed against 0·05 M Tris-HCl, pH 7·5, 0·005 M EDTA and 02 M DTT. Aliquots of 0·5 ml were stored frozen at −20°C until use.

ino acid acceptor assay

inoacylation of tRNA was measured by incorporation of radioactive amino acids o trichloroacetic acid (TCA) insoluble material using the paper disc assay of ns and Novelli (1961). The reaction was carried out in a final volume of 0·3 ml ntaining 100 mM Tris-HCl, pH 7·5, 10 mM $MgCl_2$, 10 mM KCl, 10 mM NH_4Cl, M 2-mercaptoethanol, 2 mM ATP, pH 7, 1 mM CTP, pH 7, 0·2 μCi of [^{14}C] al protein hydrolysate or [^{14}C] amino acids; 2 A_{260} units of tRNA and 0·3 mg of yme protein. The reaction mixture was incubated at 37°C for varying time iods. Aliquots were taken and applied to Whatman 1 filter paper discs. The ction was stopped by putting the discs in ice-cold 10% TCA. The discs were cessively washed with 10 and 5% TCA, ethanol and dried under infra-red lamp. dioactivity was measured in 5 ml of toluene based scintillator containing 0·4% O and 0·01% POPOP in a Beckman LS 1800 counter (channel 2).

Purity of tRNA

The tRNA preparations obtained from the DEAE-cellulose column were found to be contaminated to varying degrees with high molecular weight nucleic acids (figure 1). The unio RNA always eluted as a single peak in the standard *E. coli* tRNA region from Sephadex G-100, whereas the preparations from rat liver, cockroach and earthworm eluted in 2 peaks, one in the tRNA region and the other in the void volume (high molecular weight nucleic acid). In the case of rat liver the contamination was to the extent of 15–20%, whereas in earthworm and cockroach it was much higher, 70–75%. Hence all tRNA samples as a routine, were purified on Sephadex G-100 after DEAE-cellulose chromatography. In all these studies the second peak eluting in the *E. coli* tRNA region was pooled and used as tRNA.

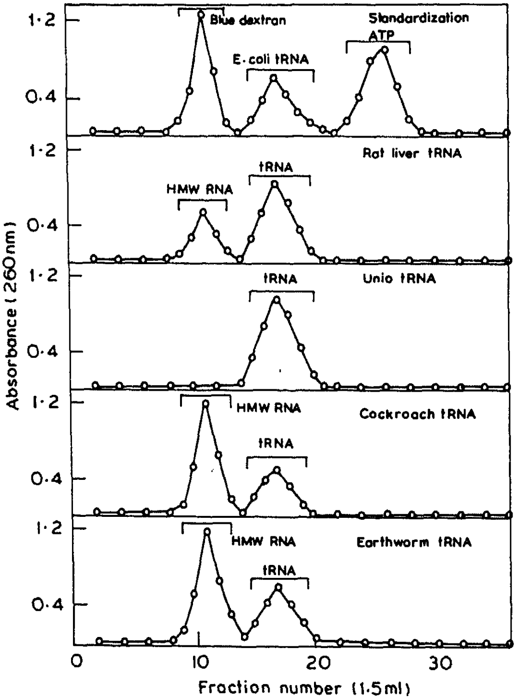


Figure 1. Gel filtration of rat liver, unio, cockroach and earthworm tRNAs on Sephadex G-100. 10 A_{260} units (2 ml) of DEAE-cellulose purified preparation was applied to a Sephadex G-100 column (34·5 × 1·3 cm). 1·5 ml fractions were collected at the rate of 20 ml/h.

Even though the same method was used for the extraction of tRNAs, it is not clear why the tRNA preparations from different animals had different degree of high molecular weight nucleic acid contaminations. It is possible that in the case of earthworm and cockroach the high molecular weight nucleic acids are complexed with some other component which makes them elute at a lower salt concentration from the DEAE-cellulose column or the phenol extraction step could be less efficient in denaturing the macromolecules in these organisms.

DNA contamination

The tRNA preparations were checked for DNA contamination based on the sensitivity of RNA to alkali hydrolysis, the DNA being stable under these conditions (Dure 1973). The gel filtration patterns indicated that all the tRNA preparations were free of any DNA contamination since after alkali hydrolysis the entire tRNA peak moved to the low molecular weight region and no alkali stable material was detected in tRNA region.

Variation of total tRNAs

A comparison of the yield of total tRNA (figure 2) from earthworm, cockroach, unio and rat liver clearly indicate that the tRNA content is much lower in the case of invertebrates as compared to an active mammalian tissue, the rat liver, when expressed on the basis of wet weight or other parameters. Among the invertebrates

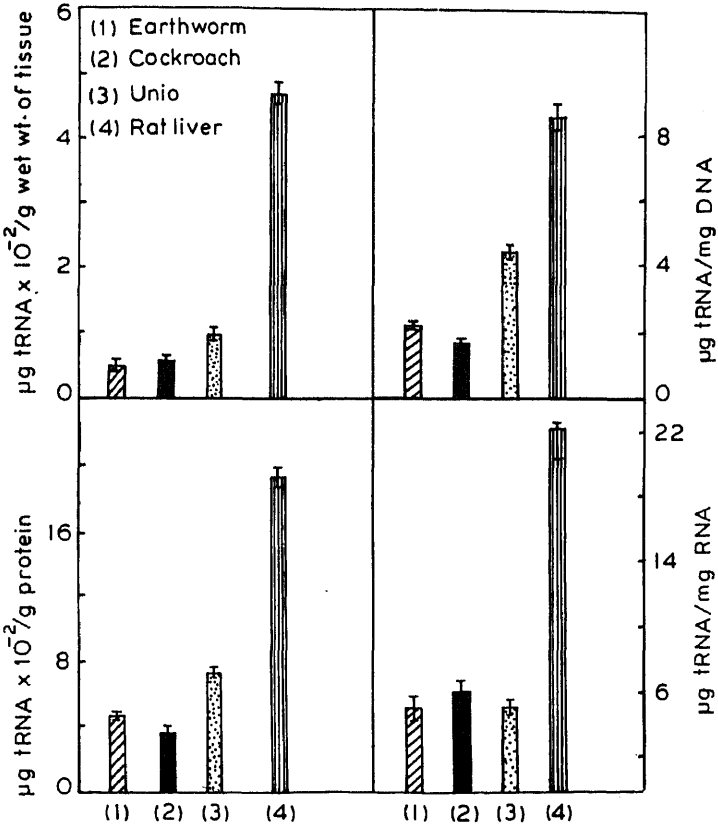


Figure 2. Variation of total tRNA content.

per gram protein. But the tRNA content seems to be more or less equal in all the 3 invertebrates when expressed per mg RNA.

Qualitative and quantitative variations of the tRNA are a reflection of the activity of the tissue under study and a higher content of tRNA may reflect a higher rate of protein synthesis. Earlier reports have shown that the tRNA content is higher in a fast developing tissue such as the chick embryo (Mayasundari *et al.*, 1974) and in a mitotic and metabolically active tissue such as the rat liver (Manjula and Mayasundari, 1981) than a post mitotic tissue such as the rat skeletal muscle (Manjula and Mayasundari, 1980). In line with this, it is possible that the rate of protein synthesis is much lower in invertebrates than the metabolically active rat liver tissue.

Variation of total amino acid acceptor capacity of tRNAs

Assay of the tRNA preparation from earthworm, cockroach, unio and rat liver with homologous enzymes, using [^{14}C] algal protein hydrolysate as a source of the total amino acids (figure 3) indicate that the rat liver and unio systems are highly active whereas those from earthworm and cockroach are totally inactive. In order to determine, whether the total inactivation of aminoacylation in, these two systems was due to the tRNA preparations from the two organisms or the aminoacyl-tRNA synthetases, aminoacylation was carried out under heterologous conditions (figure 4). The earthworm and cockroach tRNAs which were totally inactive with homologous enzymes were partially active with enzyme from heterologous sources, showing 20–25% of the activity as compared to the rat liver system, under these

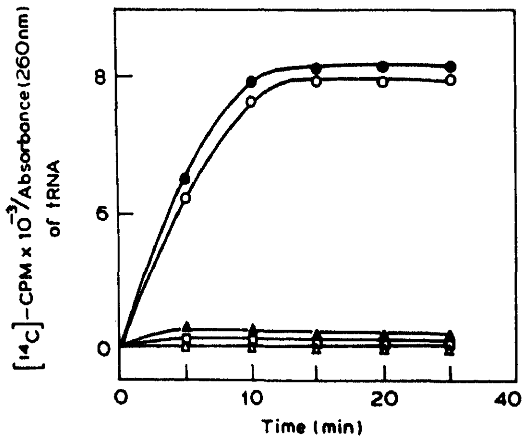


Figure 3. Amino acid acceptor activities of the tRNAs from unio (●), cockroach (▲), earthworm (□) and rat liver (○) with homologous enzymes. [^{14}C] Algal protein hydrolysate was used as the source of amino acids. (△), Without tRNA.

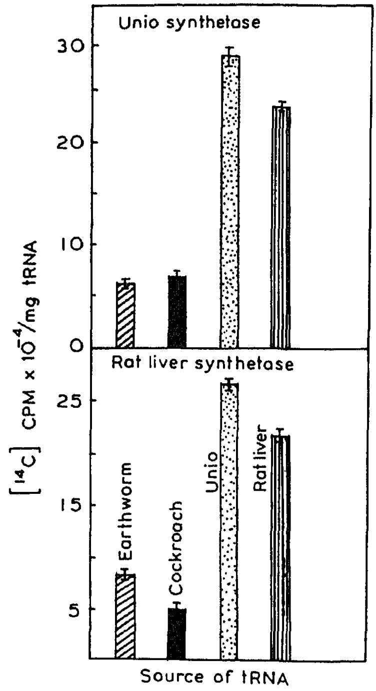


Figure 4. Total amino acid acceptor activities of different tRNAs.

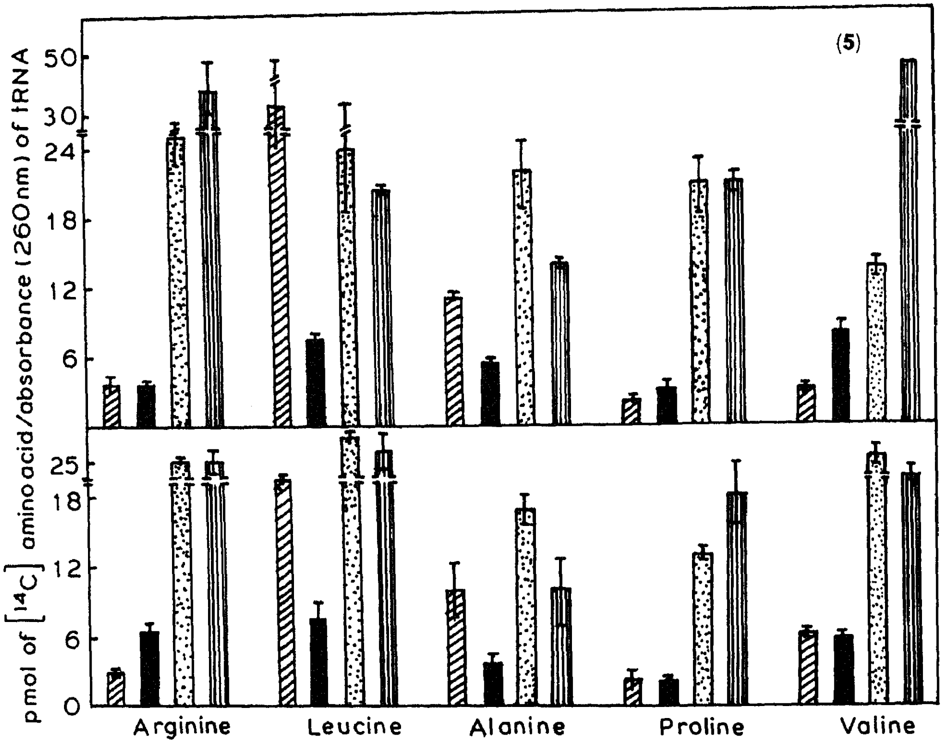
litions. On the other hand the unio and rat liver tRNAs were active with ologous and heterologous enzymes.

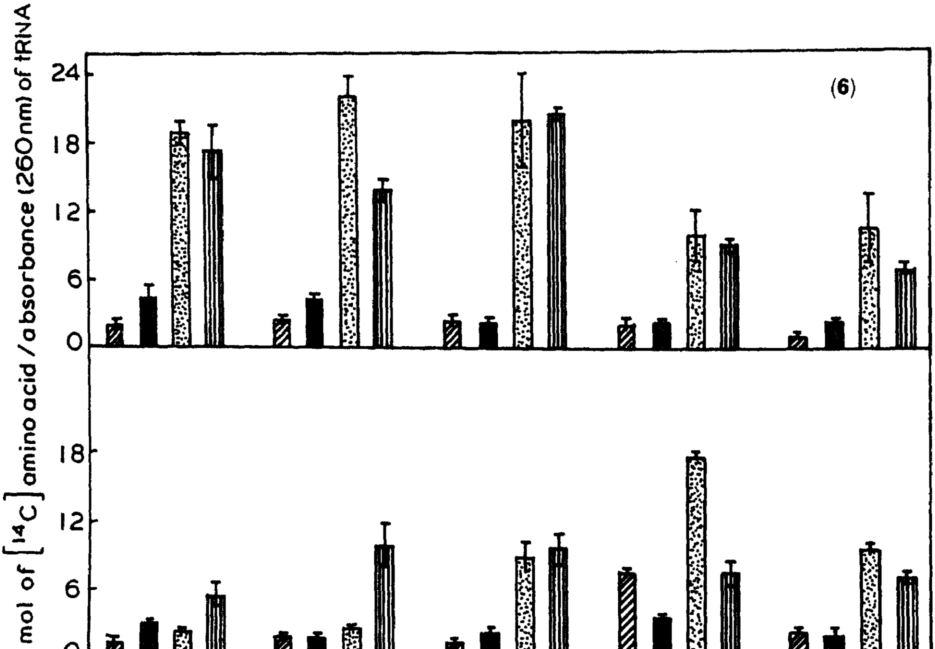
is not clear at present why earthworm and cockroach tRNAs show a low no acid acceptor activity. It is obviously not because they are assayed with mes from heterologous source, since unio which is closely related to earthworm cockroach in evolution shows equal activity when assayed with homologous me or rat liver enzyme. It is unlikely that the unio enzyme is more closely ed to the mammalian enzyme than to the earthworm and cockroach enzymes.

ation of individual aminoacyl tRNAs

res 5 and 6 compare the variation in amino acid acceptor capacities of vidual tRNAs from the 4 above mentioned organisms, when acylated with unio me or rat liver enzyme. There are significant quantitative variations observed ng the 10 amino acids studied. In agreement with the earlier results the tRNAs earthworm and cockroach in general show a low acceptor activity for most of amino acids studied. The earthworm tRNAs show quite low activities with all no acids except alanine and leucine, where the activities are on par with those of and rat liver.

ith regard to the rat liver homologous system there is a quantitative variation


(5)
pmol of [¹⁴C] amino acid/absorbance (260nm) of tRNA
50
30
24
18
12
6
25
18
12
6
0
Arginine
Leucine
Alanine
Proline
Valine


(6)
mol of [¹⁴C] amino acid/absorbance (260nm) of tRNA
24
18
12
6
0
18
12
6
0

hand show a different relative abundancy pattern. It has high concentration of leucine, arginine and valine tRNAs followed by alanine, proline, phenylalanine, tyrosine and lysine tRNAs. The acidic amino acids like aspartic acid and glutamic acid are found at very low concentrations. In mammals, liver is known to be a metabolically active organ synthesizing different types of proteins and also rich in enzymes. Hence it is difficult to correlate the variations in the levels of aminoacyl-tRNAs with the synthesis of any particular protein.

All the above results throw light on the fact that aminoacyl-tRNA synthetases from rat liver and unio are active, whereas those prepared from earthworm and cockroach are inactive. It is well known from literature that the aminoacyl-tRNA synthetase complexes are highly unstable and get inactivated rather easily during the course of purification (Soll and Schimmel, 1974). Hence various methods known to stabilize the enzyme like inclusion of glycerol, use of mild homogenizing conditions etc., were tried but without success. Since in many cases synthetases are shown to be membrane bound (Deutscher, 1981), solubilizing the enzyme using detergents were also tried but with no success. Since all attempts to stabilize and obtain an active enzyme preparation from earthworm and cockroach failed it was suspected that the enzyme preparation from these two sources may contain an aminoacylation inhibitor(s). Further work has provided support to this hypothesis and has led to the identification of aminoacylation inhibitor(s) in earthworm and cockroach enzymes (data not shown). At present it is not clear whether the low amino acid acceptor activities shown by tRNAs from earthworm and cockroach is due to the presence of such inhibitory compounds. More work is required to be done to confirm this aspect.

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Figures 5 and 6. tRNA from earthworm (▨), cockroach (■), unio (⊞) and rat liver (▥) were acylated with [^{14}C]amino acids using the aminoacyl-tRNA synthetase preparation from rat liver (top portion) and unio (lower portion). Specific radioactivities (mCi/mmol) of the [^{14}C]amino acids were, arginine (246), proline (175), glutamic acid (190), leucine (156), valine (190), lysine (288), alanine (120), tyrosine (360), aspartic acid (152) and phenyl alanine (234).

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tabolism of glycosaminoglycans in CCl_4-induced liver regeneration

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Abstract. The incorporation of $[^{35}S]$-SO_4 into glycosaminoglycans of liver *in vivo* and in liver slices and into the glycosaminoglycans associated with the hepatic plasma membrane of rats at different periods after a heavy dose of CCl_4 have been studied. The incorporation of $[^{35}S]$-SO_4 into total glycosaminoglycans decreased to as low as 40% of the control at 24 h after the administration of CCl_4 and later on increased reaching a maximum on the 4th day. The amount of $[^{35}S]$-SO_4 incorporation into heparan sulphate was also reduced to about 40% of control at 12–24 h after the onset of injury and increased thereafter reaching a maximum on the 4th day. There was only a partial reduction in the synthesis of chondroitin sulphate in the early stage of injury and then it steadily increased reaching about 3 times the control level on 4–6 days. The $[^{35}S]$-SO_4-incorporation into dermatan sulphate, after a slight initial decrease remained at the control levels. On the 8th day after the CCl_4-induced liver injury, the rate of $[^{35}S]$-SO_4-incorporation was almost equal to that in normal controls. The incorporation of $[^{35}S]$-SO_4 into hepatic plasma membrane glycosaminoglycans showed a similar change decreasing to about 35% of control at 24 h followed by an increase, reaching normal levels on the 4th day after the administration of CCl_4. About 90% of the plasma membrane glycosaminoglycans was found to be heparan sulphate. The yield of plasma membrane from normal and CCl_4-induced regenerating liver was found to be similar and therefore the results obtained were not due to difference in the yield of the membrane preparation. The data also indicate that there was no difference in the degree of sulphation. The significance of these changes in the metabolism of sulphated glycosaminoglycans particularly plasma membrane heparan sulphate in tissue regeneration has been discussed.

Keywords. Glycosaminoglycans; liver regeneration; plasma membrane heparan sulphate.

oduction

external surface of a number of different cell types have been shown to contain osaminoglycans (GAG) particularly heparan sulphate (HS) (Hook *et al.*, 1984; lagher *et al.*, 1986; Poole, 1986). HS has been shown to be associated with atocyte cell membrane, plasma membrane (PM) of ascites hepatoma and rat liver . It has been suggested that the cell surface HS may have a role in the ilation of cell proliferation, cell-matrix interaction, cell-cell communication, trol of exchange of metabolites, ions, fluids and water or in the accessibility of erlying membrane receptors to external agents.

arlier reports show that there is accumulation of GAG and collagen in long ı damaged liver (Gressner, 1980; Rauterberg *et al.*, 1981; Unnikrishnan and hakaran, 1985). Hepatic regeneration is a well known histologic symptom of onically damaged livers. For human pathology, the induction of hepatocellular

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whom all the correspondence should be addressed.

reviations used: GAG, Glycosaminoglycans; HS, heparan sulphate; PM, plasma membrane; CS, the pathobiochemistry of liver. Edward *et al.* (1980) has observed a biphasic change in chondroitin sulphate (CS) A/C and dermatan sulphate (DS) in liver regeneration after partial hepatectomy, but there was no change in the amount of HS in liver. During the regeneration of mouse liver after partial hepatectomy, the synthesis of sulphated GAG was found to increase following a pattern similar to that of DNA synthesis (Yoshiharu, 1981). However, it is not known clearly whether there is any relationship between the metabolism of GAG in injured liver and hepatic regeneration though it has been suggested that the presence of HS at the surface of cell decrease their tendency to proliferate and that this effect is diminished in growing cells by an increase in the content of less highly sulphated GAG (Edward *et al.*, 1980). In view of the accumulation of GAG in liver injury which leads to fibrosis and cirrhosis and the suggested role of cell surface GAG especially HS in cell proliferation, the study of changes in GAG, particularly PM GAG, in liver undergoing regeneration is significant. A single oral dose of CCl_4 may lead to severe liver damage which is followed by rapid proliferation of the surviving cells (Levy *et al.*, 1959). The regenerating liver after intoxication with CCl_4 has been used to study the changes in the metabolism of GAG particularly PM GAG.

Materials and methods

Materials

Radioactive sulphate used was from the Bhabha Atomic Research Centre, Bombay. Chondroitinase ABC, chondroitinase AC, Sephadex G-25 etc., were from Sigma Chemicals Co., St. Louis, Missouri, USA. All other reagents used were of analytical or equivalent grade.

Methods

Treatment of rats: Male sprague Dawley rats weighing 180–200 g fed Gold Mohur diet *ad libitum* were used. The animals received a single dose of 0·5 ml/100 g body weight of CCl_4 in ground nut oil (1 : 1 v/v) through an intra gastric tube. The rats were killed at different periods after the treatment and the livers were taken in ice-cold containers. Control rats, receiving oil alone were killed along with experimental rats.

Preparation of PM from liver: A 10% tissue homogenate was prepared by homogenising liver in a glass-glass homogeniser and the PM was partially purified by the procedure of Neville (1968) except the last linear gradient centrifugation as modified by Pohl *et al.* (1971).

Isolation and characterisation of labelled GAG of PM: Rats were injected intra peritoneally 0·5 mCi of $[^{35}S]$-Na_2SO_4 3 h before they were killed and PM fraction was prepared from livers as described above. $[^{35}S]$Labelled polysaccharides were isolated from the PM by digestion with papain after delipidation with cold acetone lescribed before. (Unnikrishnan and Sudhakaran, 1985). The [^{35}S]labelled saccharides were finally precipitated with 3 vols of 96% alcohol containing ₀ potassium acetate in the presence of 0·25 mg/ml bovine aortic CS as carrier.

igestion of the [^{35}S]-polysaccharide with chondroitinase ABC and AC and ration of the products by paper chromatography was carried out according to › *et al.* (1968). Deaminative degradation of the sulphated polysaccharide was ied out by the method of Shiveley and Conrad (1970).

ıe total [^{35}S]labelled GAG were isolated from dry defatted tissue after papain stion and analysed as above.

rporation of [^{35}S]*-SO$_4$ into GAG by liver slices in vitro*: The rate of synthesis ılphated GAG in liver of experimental animals was studied by following the rporation of [^{35}S]-SO$_4$ into GAG *in vitro*. The liver slices prepared from ıals at different time after CCl$_4$ administration were used. The details of the edure are given before (Unnikrishnan and Sudhakaran, 1985).

iltration over Sephadex G-25: The amount of nitrous acid susceptible material determined by gel filtration of the [^{35}S]labelled material before and after ɔus acid degradation over Sephadex G-25 (1·8 × 50 cm) equilibrated in pyridine-ate buffer (0·2 M, pH 5·5). Fractions were collected and the radioactivity in each ion was determined.

y of enzymes

activity of 5′-nucleotidase (EC 3·1·3·5) was determined by the method of ansky and Schwartz (1963). The activity of glucose-6-phosphatase (G-6-P) (EC ·9) was determined by the method of Swanson (1955) with the addition of M EDTA. The activity of succinate-cytochrome-C reductase was determined by procedure of Fleischer and Fleischer (1967). Protein was estimated by the ıod of Lowry *et al.* (1951).

lts

ıinistration of a high dose of CCl$_4$ caused severe liver damage. At a dose of ıl/100 g body weight the mortality rate was 40% (mostly within 24 h after the ment). The levels of serum glutamate: pyruvate transaminase which reflects the ıt of liver damage, increased rapidly. Its maximum activity was 100 times the rol level two days after administration of CCl$_4$. Its activity decreased thereafter reached near normal values, 5 days after treatment.

rporation of [^{35}S]*-SO$_4$ into GAG by liver slices in vitro*

synthesis of GAG by regenerating liver of animals at different time intervals receiving a heavy dose of CCl$_4$ was studied *in vitro* by determining the amount ^{5}S]-SO$_4$-incorporated into sulphated GAG by liver slices. The results are given gure 1.

ıe incorporation of [^{35}S]-SO$_4$ into total liver GAG decreased rapidly in

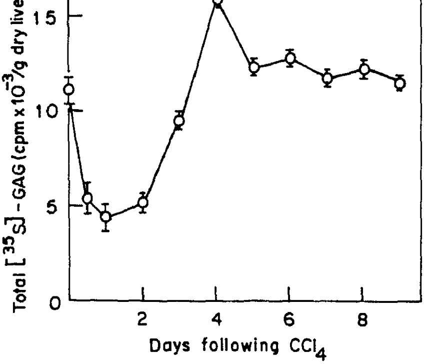

Figure 1. Incorporation of [^{35}S]-SO$_4$ into total GAG by liver slices from rats undergoing hepatic regeneration at different periods after a heavy dose of CCl$_4$.

Slices were prepared from normal control and regenerating rat liver at different periods after a heavy dose of CCl$_4$ and incubated in a medium containing [^{35}S]-Na$_2$SO$_4$ at 37°C for 4 h. The total [^{35}S]-GAG were isolated from each sample at the end of the incubation and the radioactivity was measured. Average of the values from 4 experiments. In slices prepared from control rat livers at different periods (1, 2, 5 and 9th day) there was no difference in the incorporation of radioactivity into GAG, the average of which is shown as 0 time. Vertical bars indicate the range of values.

response to a single dose of CCl$_4$. It was reduced to as low as 40% of the control at 24 h after the administration of CCl$_4$. Later on the rate of incorporation increased reaching a maximum on the 4th day; 6 to 7 days after the administration of CCl$_4$, the rate of [^{35}S]-SO$_4$-incorporation was almost similar to that in normal livers.

An analysis of the different [^{35}S]-GAG showed similar biphasic change (figure 2); an initial decrease followed by an increase was observed in the case of HS. [^{35}S]-SO$_4$-incorporation into HS reached a minimum at 12–24 h after the administration of CCl$_4$ and increased thereafter reaching a maximum on 4th day. However the rate of [^{35}S]-SO$_4$-incorporation into CS followed a different pattern. There was a very small reduction in the [^{35}S]-SO$_4$-incorporation in the early stages, at 12 h after

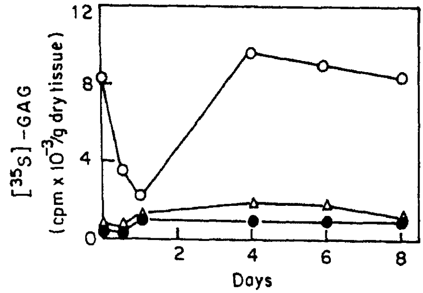

Figure 2. Incorporation of [^{35}S]-SO$_4$ into individual GAG by liver slices from rats undergoing hepatic regeneration at different periods after a heavy dose of CCl$_4$.

The total [^{35}S]-GAG isolated from each sample of each group were pooled (see legends to figure 1) and the amount of different [^{35}S]-GAG in each pooled sample were analysed. The values given are the mean of duplicate analysis. (○), HS; (△), CS; (●), DS.

ury, but thereafter it steadily increased reaching about 3 times the normal level 4–6 days. It was similar to normal levels on the 8th day. The incorporation into ; was also slightly reduced initially and thereafter reached normal level. These ults suggest that the production of CS and DS is affected in a different manner.

:orporation of $[^{35}S]$-SO_4 *into sulphated GAG in vivo*

e synthesis of GAG by regenerating liver of rats at different time intervals after a avy dose of CCl_4 was also studied by injecting $[^{35}S]$-SO_4 intraperitoneally and lating the total sulphated GAG. The results are given in figure 3.

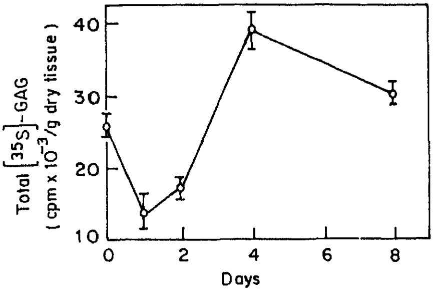

Figure 3. Incorporation of $[^{35}S]$-SO_4 into total GAG in liver *in vivo* in rats undergoing hepatic regeneration at different periods after a heavy dose of CCl_4.

CCl_4 was administered to rats by an intra gastric tube and at various times thereafter 0·5 mCi of $[^{35}S]$-Na_2SO_4 (carrier free) was injected intraperitoneally. Rats were killed 3 h after administration of $[^{35}S]$-sulphate and the total $[^{35}S]$-GAG were isolated as described in methods. The radioactivity of total $[^{35}S]$-GAG was measured. The values are the average of 4 to 5 experiments. Vertical bars indicate the range of values. Control rats at different periods (1, 4 and 8th day) were used and the mean value was taken.

Administration of a single dose of CCl_4 to rats resulted in a rapid decrease the incorporation of $[^{35}S]$ sulphate into total liver GAG. The rate of :orporation was reduced to 50% of the untreated control at 24 h after the onset injury and thereafter it increased reaching a maximum on the 4th day. On the ı day after CCl_4 treatment the amount of $[^{35}S]$-GAG was similar to that of the treated controls.

›lation of PM $[^{35}S]$ *HS from regenerating rat liver*

order to study whether the alteration in the synthesis of GAG in regenerating er as evidenced by altered rate of incorporation of $[^{35}S]$-SO_4 both *in vitro* and *vivo*, has affected the PM polysaccharides, the PM was isolated from the generating liver. The purity of the isolated membrane was assessed by termining the activity of marker enzymes.

The specific activities and yield of 5′-nucleotidase, G-6-P and succinate-tochrome C reductase, the marker enzymes for PM, microsomes and tochondria respectively are given in table 1. The specific activity of 5′-cleotidase increased about 14-fold on an average in PM preparation compared

Table 1. Preparation of PM from normal and CCl_4 induced regenerating rat liver. Activity and yield of marker enzymes.

	Time after CCl_4 administration		
	Control (0 h)	48 h	96 h
Homogenate			
Sp. activity (units/mg protein)			
5′-Nucleotidase	1·84 ± 0·16	1·76 ± 0·14	1·81 ± 0·20
Succinate-cytochrome C reductase	0·87 ± 0·06	0·91 ± 0·1	0·94 ± 0·1
G-6-P	2·4 ± 0·26	2·15 ± 0·24	2·31 ± 0·3
Membrane			
Sp. activity (units/mg protein)			
5′-Nucleotidase	25·8 ± 1·8	24·9 ± 1·4	27·15 ± 2·4
Succinate-cytochrome C reductase	0·53 ± 0·08	0·5 ± 0·06	0·48 ± 0·09
G-6-P	1·9 ± 0·3	1·6 ± 0·24	1·8 ± 0·25
Membrane protein (mg/g)	2·21 ± 0·09	2·05 ± 0·11	2·16 ± 0·14
Yield (%)			
5′-Nucleotidase	21·1	19·8	20·6
Succinate-cytochrome C reductase	0·90	0·77	0·83
G-6-P	1·22	1·09	1·18
Purification			
5′-Nucleotidase	14·02	13·69	15·00
Succinate-cytochrome C reductase	0·6	0·55	0·51
G-6-P	0·79	0·75	0·78

Homogenate and PM were prepared from normal control and CCl_4 treated rats at 48 and 96 h after the administration of 0·5 ml CCl_4 and assays were carried out as described in methods. Specific activities of 5′-nucleotidase and G-6-P are expressed as μmol of P_i/h/mg protein. Succinate-cytochrome C reductase activity is expressed as μmol of succinate oxidised/h/mg protein. Average values of 3–4 different analysis ±SE are given. Yields of different enzymes in the membrane prepartion are calculated as the percentage of the total activity of the respective homogenate. Degree of purification is calculated as a ratio of the average specific activity of the preparation to that of the homogenate.

with the homogenates. The recovery of the PM 5′-nucleotidase, calculated as a fraction of the total activity of the homogenate and its specific activity were similar in normal and CCl_4 treated rat livers. The average recovery of PM 5′-nucleotidase was 19–20%. There was no significant difference in the specific activities of other marker enzymes in the PM fraction isolated from rat liver at different times after CCl_4 treatment. About 1% of the total G-6-P activity and 0·8% of succinate-cytochrome C reductase activity were obtained in the PM fraction indicating microsomal and mitochondrial contamination which apparently was not different in membrane preparations from normal and CCl_4 treated rat livers. Further, there was no significant difference in the amount of total protein in the membrane prepared from normal and intoxicated rats. Although 5′-nucleotidase was only 14-fold enriched, the relative purification of liver PM preparation did not differ significantly between respective normal and CCl_4 treated rats.

The amount of $[^{35}S]$-SO_4 incorporated into the PM polysaccharides was reduced to about 35% of that of the untreated control at 24 h after the CCl_4

atment after which the rate of incorporation increased reaching a level equal to t of the untreated control on the 4th day (figure 4). On the 8th day after CCl_4 atment there was no difference in the amount of [^{35}S]polysaccharides associated h the PM when compared with the control. About 90% of the S]polysaccharide of the liver PM of the untreated control rat was resistant to ndroitinase ABC digestion. More than 90% of this material could be degraded treatment with nitrous acid. The [^{35}S]polysaccharide isolated from the PM of rats 4 days after CCl_4 treatment was also found to be resistant to ndroitinase ABC digestion and susceptible to nitrous acid degradation to a ilar extent as the control (figure 5). The [^{35}S]polysaccharides isolated from the

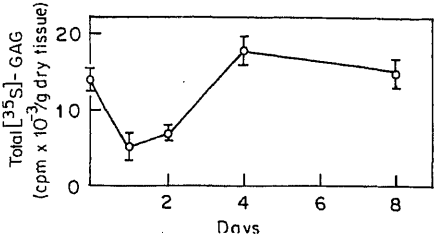

Figure 4. Hepatic PM [^{35}S]-GAG of rats undergoing liver regeneration at different periods after a heavy dose of CCl_4.

Total liver GAG of rats undergoing liver regeneration were labelled with [^{35}S]-SO_4 as described in legend to figure 3. The PM were isolated from the livers of rats at different periods after the administration of CCl_4 and the total [^{35}S]-GAG associated with the PM were isolated as described in methods. The values given are the average of 3–5 experiments. The vertical bars show the range of values.

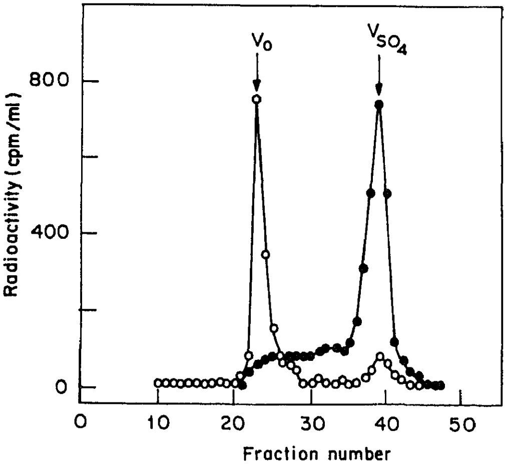

Figure 5. Deaminative degradation of [^{35}S]-GAG isolated from hepatic PM.

[^{35}S]-GAG were prepared from the hepatic PM of rats 4 days after a heavy dose of CCl_4 by intraperitoneal injection of [^{35}S]-Na_2SO_4 as described in the text. Samples were applied on Sephadex G-25 column (1·8 × 50 cm) in 0·2 M pyridine-acetic acid pH 5·5 before (○) and after (●) nitrous acid degradation at pH 1·5. The fractions were assayed for radioactivity. The average fraction size was 1·65 ml. Vo and V_{SO_4} are the elution volumes for dextran blue and [^{35}S]-Na_2SO_4 respectively. Similar elution pattern was obtained for nitrous acid degradation of [^{35}S]-GAG from normal rat hepatic PM and that from rats 2 days after CCl_4 administration.

PM of normal and regenerating rat liver were eluted from Dowex AG 1 × 2 at a salt concentration of 1·2 M in both cases indicating that the major GAG is HS (figure 6).

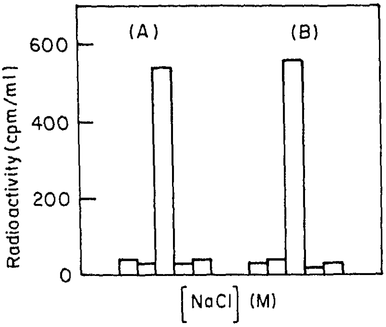

Figure 6. Ion exchange chromatography of PM [^{35}S]-HS on Dowex AG 1 × 2.

[^{35}S]-GAG associated with the hepatic PM of normal control and CCl_4 induced regenerating rat liver on 4th day after administration of CCl_4 were isolated as described in the text. The [^{35}S]-GAG were applied on Dowex AG 1 × 2 (0·5 × 5 cm) and eluted with 2 × 2 ml each of different concentration of sodium chloride. The radioactivity shown from left to right are the activity eluted with 0·5, 1, 1·2, 1·5 and 2·5 M NaCl respectively. (A), PM [^{35}S]-GAG from normal control; (B), PM [^{35}S]-GAG from rat liver on 4th day followed by CCl_4 intoxication. Similar elution pattern was observed for the [^{35}S]-GAG isolated from PM on 2nd day after a single dose of CCl_4.

Discussion

The results of the investigations described above show that CCl_4 induced liver regeneration is associated with biphasic changes in the metabolism of sulphated GAG. During the early stages of the liver injury, rate of synthesis of sulphated GAG is decreased to about 40% of that of the untreated control and later on there is a rapid increase reaching a maximum on the 4th day. The biphasic change in the sulphated GAG was reflected more in the amount of HS. There was only a partial reduction in the synthesis of CS A/C in the early stages of liver injury lasting only for a few hours and then it steadily increased reaching about 3 times the control level on 4–6 days. The synthesis of DS was also slightly reduced initially and then reached the normal levels. A similar biphasic response in the metabolism of total as well as individual GAG has been reported in thioacetamide and glucosamine treated rats by Gressner *et al.* (1981), the rate of [^{35}S]-SO_4 incorporation into HS being the lowest at 2 h and maximum at 24–48 h, after the administration of the drug. The difference in the peak periods showing the biphasic response in [^{35}S]-SO_4 incorporation may be probably due to difference in the degree of injury caused by these different drugs. A biphasic change in the amount of total sulphated GAG has also been reported in liver regeneration after partial hepatectomy (Edward *et al.*, 1980). Control experiments showed that diet restriction was not a major factor contributing to a change in the metabolism of GAG after a heavy dose of CCl_4.

Previous studies have shown that the addition of Bt_2 cAMP to primary cultures lar results have also been observed in liver tissue slices *in vitro* (Gressner and ıltz, 1981). The amount of cAMP in the homogenate prepared from the livers of after CCl_4 intoxication was found to increase to a peak level during the early es of the liver injury (Mourelle and Rubalcave, 1981). Therefore the decrease in]-SO_4 incorporation into HS during the early stages of CCl_4 intoxication may ably be due to the action of cAMP on hepatocytes, which in culture has been vn to synthesise almost exclusively HS (Prinz *et al.*, 1980). CS A/C and DS h might be synthesised mostly by other types of cells, may be affected rently.

isceptibility to nitrous acid degradation, ion exchange chromatography over vex AG 1 × 2 and resistance to chondroitinase ABC digestion showed that the]labelled polysaccharide associated with the PM isolated from normal and treated animals was HS. The [^{35}S]-SO_4-incorporated into PM HS showed a ction reaching a minimum at 24 h after the onset of liver injury, thereafter ly increased and reached a maximum on the 4th day indicating that the PM has been affected during CCl_4 induced liver regeneration. This difference rved is apparently not due to difference in the yield of PM from CCl_4 treated ıals. The [^{35}S]polysaccharide isolated from the PM of the animals on 2nd and days after CCl_4 treatment was also found to be resistant to chondroitinase ABC stion and susceptible to nitrous acid degradation to a similar extent (90%) as control. This indicates that there has been no change in the nature of the saccharide associated with the PM even though the actual amount of HS that sociated with the membrane has been affected. These results showing HS as the ominant sulphated polysaccharide detected in the protease digest of the PM of ıal and regenerating rat liver were similar to those reported by Akasaki *et al.* 5) and Oldberg *et al.* (1979). However these data differ from the findings of ma *et al.* (1975) who claimed that CS–B occurred in the PM of normal rat s in addition to HS at an approximate ratio of 1:30 and that its content ased in regenerating livers reaching nearly the level of HS 28 h after tectomy. It is rather difficult to explain this inconsistency.

ıe possibility that the change in the structure of HS is related with neoplastic sformation is supported by the finding that HS isolated from an ascites toma and the HS produced by a hepatoma cell line have been shown to have a r degree of sulphation than the HS isolated from normal rat liver and cultured ıepatocytes. No difference in the degree of sulphation of PM HS isolated from ıal and CCl_4 induced regenerating liver could be observed in the ion exchange matographic experiments reported above.

ıe highest rate of regeneration has been observed at 48–72 h following CCl_4 inistration in rats and probably indicates new cell formation (Levy *et al.*, 1959). pid decrease in PM HS has been observed in our study in early stages of the y which increases to a maximum in the later stages. One of the views regarding role of cell surface GAG in cell proliferation is that depletion of cell surface eo—HS might trigger the liver cell proliferation, by facilitating the influx of cell ice membrane associated Ca^{2+} into the cytosol (Berridge, 1975). Another ested possibility involves the interaction with different binding capacity of cell ice GAG with proteinases which could be involved in the control of cell feration (Kindness *et al.*, 1979).

owever, in the absence of adequate information establishing the stages of regeneration and the fraction of cells undergoing regeneration and in view of the reported finding that different population of hepatocytes in liver divide asynchronously (Rabes, 1978), it is rather difficult to correlate these temporal biphasic changes in PM HS with the regeneration of the cells. However, it is possible that HS has a significant role in the proliferation of liver cells.

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Detoxification of mercury and organomercurials by nitrogen-fixing soil bacteria

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Abstract. Minimal inhibitory concentration values of $HgCl_2$ and 5 organomercurials were determined against 24 mercury-resistant N_2-fixing soil bacteria previously isolated from soil and identified in our laboratory. These bacterial strains also displayed multiple antibiotic resistant properties. Typical growth pattern of a highly mercury-resistant *Beijerinckia* sp (KDr_2) was studied in liquid broth supplemented with toxic levels of mercury compounds. Four bacterial strains were selected for determining their ability to volatilize mercury and their Hg-volatilizing capacity was different. Cell-free extracts prepared from overnight mercury-induced cells catalyzed Hg^{2+}-induced NADPH oxidation. Specific activities of Hg^{2+}-reductase which is capable of catalyzing conversion of $Hg^{2+} \rightarrow Hg(o)$ of 10 Hg-resistant bacterial strains are also reported.

Keywords. Mercury and organomercurials; N_2-fixing soil bacteria; volatilization; cell-free extracts.

Introduction

Mercury and organomercurial resistance in microbial cells is determined by plasmids harboring mercury-resistance genetic factors which code for synthesis of inducible enzymes such as mercury reductase and organomercurial lyase (Schottel *et al.*, 1974; Clark *et al.*, 1977; Izaki, 1978; Summers and Silver, 1978; Khesin and Karasyova, 1984; Lomovskaya *et al.*, 1985). Concerted action of both the enzymes are needed to breakdown organomercurials such as phenylmercuric acetate (PMA), methyl mercuric chloride and ethyl mercuric chloride, thimersol etc., ultimately to Hg(o) which volatilizes out of the system (Schottel *et al.*, 1974; Summers and Silver, 1978; Tezuka and Tonomura, 1978; Hayakawa and Nonomura, 1983). Organomercurial lyase catalyzes hydrolysis of carbon-mercury bonds to liberate Hg^{2+} which is reduced by mercury reductase in the presence of NADPH and sulfhydryl groups (Schottel, 1978; Nakahara *et al.*, 1985; Sandstrom and Lindskog, 1987). Narrow-spectrum Hg-resistant organisms harbor only Hg-reductase, whereas organisms having 'broad-spectrum' mercury-resistance possess both the enzymes (Summers and Silver, 1978). Investigations of mercury-resistance have been carried out in both narrow and broad-spectrum mercury-resistant bacterial strains (Schottel *et al.*, 1974).

There is very little information about mercury-resistance in N_2-fixing micro-organisms. In this context, studies by Prasad *et al.* (1986) on the biological effect resistance spectra, antibiotic resistance spectra, growth of cells with or without mercury compounds, volatilization of mercury by growing cells and cell-free extracts as well as inducibility of mercury volatilizing enzymes by $HgCl_2$ in several N_2-fixing Hg-resistant organisms isolated from soil collected from different agricultural farms of West Bengal.

Materials and methods

All chemicals and reagents used in this study were of analytical grade (E. Merck, Germany and British Drug House, UK). $HgCl_2$, organomercurials and NADPH (tetra sodium salt) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. The antibiotics were kindly supplied by the Central Drugs Laboratories, Calcutta.

Isolation of bacterial strains

Different soil samples collected from agricultural farms were screened and Hg-resistant N_2-fixing bacteria isolated on N_2-free mannitol agar medium supplemented with 8·4 μg/ml of $HgCl_2$, a concentration of $HgCl_2$ that can inhibit the growth of both Gram positive and Gram negative bacteria. The isolated bacterial colonies were purified and identified according to the method of Bergey (1974).

Determination of minimal inhibitory concentration of $HgCl_2$ and other organomercurials against N_2-fixing isolates

Minimal inhibitory concentration (MIC) values of $HgCl_2$ and organomercurials such as merbromin, thimersol, *p*-hydroxymercuribenzoate (PHMB), fluorescein mercuric acetate (FMA) and PMA against these organisms were determined using filter paper discs (4 mm diameter) containing suitable concentrations of Hg-compounds on nutrient agar plates (Schottel *et al.*, 1974). Concentrations of $HgCl_2$ and organomercurials except PMA were 12·5, 25, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800 nmol and for PMA the concentrations were 5, 10, 12·5, 25, 50, 75, 100, 150, 200 nmol. Bacterial strains were considered sensitive to $HgCl_2$ and organomercurials (except PMA) if zone of inhibition of growth by agar disc method appeared at 12·5 nmol per disc and sensitive to PMA if at 5 nmol per disc of PMA zone of inhibition of growth appeared.

Determination of antibiotic resistance spectra of Hg-resistant N_2-fixing soil bacteria

Antibiotic sensitivity tests for the organisms were performed according to the methods of Griffith (1970) using the following concentrations of antibiotic in filter paper discs: ampicillin (10 μg), carbenicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), neomycin (30 μg), penicillin G (10 U), streptomycin (10 μg), sulphadiazine (300 μg) and tetracycline (30 μg).

wth of mercury-resistant N_2-fixing bacteria in liquid broth containing mercury pounds

wth of *Beijerinckia* sp (strain KDr$_2$) was examined in nutrient broth lemented with $0{\cdot}62 \times 10^{-4}$ M, $1{\cdot}24 \times 10^{-4}$ M and $1{\cdot}86 \times 10^{-4}$ M HgCl$_2$, $\times 10^{-4}$ M PMA and $0{\cdot}45 \times 10^{-4}$ M thimersol respectively. Inocula were ared separately by growing the organism in nutrient broth containing 10 μM Cl$_2$, PMA and thimersol respectively. The cultures were grown overnight on a ry shaker (200 rpm) and diluted 1 : 10 times with fresh sterile media and the cury compounds were added to yield the concentration of HgCl$_2$, PMA and ersol as mentioned above. The cultures were incubated with shaking and vth followed turbidimetrically at 660 nm.

tilization of mercury by mercury-resistant N_2-fixing soil isolates

nis experiment 4 bacterial strains were employed. *Beijerinckia* sp (strain KDr$_2$) *Azotobacter* sp (strain TDr$_1$) were 'broad-spectrum' Hg-resistant, *Azotobacter* strain KPr$_4$) was 'narrow-spectrum' Hg-resistant while *Azomonas* sp (strain r$_2$) was weakly Hg-resistant. In the control flask, 3·333 mg HgCl$_2$ was added to ml of nutrient broth. In the experimental flasks an overnight culture of the erial cells was diluted (1 : 10) with nutrient broth to a final volume of 200 ml and 3 mg HgCl$_2$ were added. The organisms were grown with shaking at 32°C on a ry shaker up to 12 h and the control flask was also shaken similarly. Thereafter cells were harvested by centrifugation at 6,000 *g* for 10 min at 0–4°C and ed 3 times with deionized water, weighed amount of wet cells, 1 ml of the rnatant after each cell harvesting and 1 ml of control medium containing l$_2$ were separately taken in 50 ml volumetric flask and digested to bring all the cury into ionic forms and the mercury contents of the solutions so prepared then measured by atomic absorption spectrometry technique (Brandenberger Bader, 1967; Hatch and Welland, 1968; Ebdon and Ward, 1987).

aration of cell-free extracts for Hg-reductase

-free extracts of these organisms were prepared following the method of mers and Silver (1972). Cells were grown overnight in nutrient broth at 32°C rotary shaker (200 rpm) supplemented with 10 μM HgCl$_2$ for the induction of eductase. The bacterial cultures were diluted 10-fold with the same medium concentration of HgCl$_2$ was maintained at 10 μM. A third addition of 10 μM l$_2$ was made at early log phase. Cells were harvested at late log phase by rifugation at 0–4°C and washed 3 times with cold 50 mM sodium phosphate r (pH 7·35). Washed cells were disrupted by grinding with sea sand in a mortar pestle at 4°C and suspended in the same cold buffer and centrifuged at 0 *g* for 30 min at −4°C. Most of the Hg-reductase activity in the supernatant precipitated with 30–50% $(NH_4)_2SO_4$ cut at 4°C and the precipitate was lved in minimum volume of same buffer containing 0·25 mM Na$_2$-EDTA and dialysed overnight at 4°C. The dialysates were used for the assay of Hg-ctase activities. Hg-reductase activity was determined measuring Hg^{2+}-induced PH oxidation spectrophotometrically at 340 nm (Komura *et al.*, 1971).

The reaction mixture contained: 5 mM Na_2-EDTA, 2 mM $MgCl_2$, 1 mM sodium thioglycolate, 30 μM $HgCl_2$ and 0·15 mM NADPH in 50 mM sodium phosphate buffer (pH 7·35). Suitable volumes of (10–100 μl) cell-free extracts were used to follow the reaction kinetics uniformly in a final volume of 1 ml. One unit of Hg-reductase activity was defined as the amount of enzyme that oxidised 1 μmol of NADPH per min in the presence of $HgCl_2$.

Results

Table 1 represents mercury, organomercurials and antibiotic resistance spectra of 24 N_2-fixing bacterial strains. Among these strains TDr_1 (*Azotobacter* sp), TDr_2 (*Azotobacter* sp), KDr_1 (*Beijerinckia* sp), KDr_2 (*Beijerinckia* sp), KDr_3 (*Azotobacter* sp), KDr_4 (*Beijerinckia* sp), KDr_5 (*Beijerinckia* sp), KDr_6 (*Beijerinckia* sp), KPr_2 (*Azotobacter* sp) were resistant to all the Hg-compounds (table 1). Strains KDr_1, KDr_2 and KDr_5 exhibited very high resistance to Hg-compounds and all these strains belonged to the genus *Beijerinckia.* It is interesting to note that *Azotobacter* sp T_4 is resistant to $HgCl_2$ and all mercurials except thimersol. *Azotobacter* sp T_1, *Azotobacter* sp T_2, *Azotobacter* sp T_3 and *Bacillus* sp FSr_5 displayed resistance to $HgCl_2$, merbromin, PHMB and FMA but they were highly sensitive to PMA and thimersol. *Azotobacter* sp MCr_1 and *Azotobacter* sp KPr_4 were resistant to all the Hg-compounds but were sensitive to merbromin and thimersol. The bacterial strains which were sensitive to all the Hg-compounds were strains RCr_1, RCr_2 and TPr_3 and all of them belonged to the genera *Azotobacter*. *Azomonas* sp MCr_2, *Azotobacter* sp MPr, *Azotobacter* sp BNr_1, *Azotobacter* sp BNr_3 and *Bacillus* sp PSr_7 were resistant to $HgCl_2$, PHMB and FMA but exhibited sensitivity to other mercurials. All the 24 bacterial strains possessed multiple antibiotic resistant properties.

Figure 1 represents the growth of *Beijerinckia* sp KDr_2 in the presence of graded concentration of $HgCl_2$. Growth pattern of $HgCl_2$-treated cells were similar to that of control cells without $HgCl_2$. But with increase of $HgCl_2$ concentration the lag in growth of the organism also showed a gradual increase.

Figure 2 shows the growth of *Beijerinckia* sp KDr_2 exposed to $0{\cdot}50 \times 10^{-4}$ M PMA and $0{\cdot}45 \times 10^{-4}$ M thimersol respectively. The organism manifested a lag of 30 min in both cases and growth of the cells were nearly same.

Table 2 shows the pattern of volatilization of mercury by different Hg-resistant N_2-fixing soil bacteria. In the control without organism 7·14% of total $HgCl_2$ was lost from the medium during 12 h incubation at 32°C. *Azomonas* sp MCr_2 which was weakly Hg-resistant volatilized only 10·18% of total $HgCl_2$ from the culture media during the same period but mercury bound per gram cell mass was highest in this strain *i.e.*, 178 μg. *Beijerinckia* sp KDr_2 showed highest volatilizing capacity *i.e.*, 83·15%. Other strains TDr_1 and KPr_4 also showed high mercury volatilizing capacity of 78·50% and 67·76% respectively. But in all the 3 organisms mercury bound per gram cell mass was lower compared to strain MCr_2. There are reports that Hg-resistant bacterial cells can volatilize mercury from liquid broth containing $HgCl_2$ (Komura and Izaki, 1971; Hansen *et al.*, 1984; Nakamura *et al.*, 1986; Bhattacharyya and Mandal, 1987).

ain	$HgCl_2$	Mb	Tm	PHMB	FMA	PMA	*Antibiotic resistance spectra*
zotobacter sp T_1	25	50	12·5	100	100	5	A, Cb, Cm, Em, Km, Pn, Su, Tc
zotobacter sp T_2	25	25	12·5	200	50	5	Cb, Cm, Em, Nm, Pn, Su, Tc
zotobacter sp T_3	25	25	12·5	200	100	5	A, Cb, Cm, Em, Km, Nm, Pn, Sm, Su, Tc
zotobacter sp T_4	50	25	12·5	150	50	10	A, Cb, Cm, Em, Nm, Pn, Su, Tc
zotobacter sp TDr_1	100	150	50	200	150	12·5	Cm, Em, Pn, Su
zotobacter sp TDr_2	50	25	25	100	25	10	A, Cb, Em, Km, Pn, Su, Tc
zotobacter sp MCr_1	25	12·5	12·5	50	25	10	A, Cb, Cm, Em, Km, Nm, Pn, Sm, Su, Tc.
zomonas sp MCr_2	25	12·5	12·5	50	25	5	A, Cb, Cm, Em, Pn, Tc
zotobacter sp RCr_1	12·5	12·5	12·5	12·5	12·5	5	A, Cb, Em, Nm, Pn, Su, Tc
zotobacter sp RCr_2	12·5	12·5	12·5	12·5	12·5	5	Cb, Cm, Em, Nm, Pn, Su, Tc
zotobacter sp MPr	25	12·5	12·5	50	25	5	A, Cb, Cm, Em, Km, Nm, Pn, Sm, Su, Tc
eijerinckia sp KDr_1	200	600	100	200	600	25	Cm, Em, Nm, Pn, Su
eijerinckia sp KDr_2	600	800	150	300	800	150	Cb, Em, Nm, Pn, Su
zotobacter sp KDr_3	100	150	50	50	100	12·5	A, Em, Pn, Tc, Su
eijerinckia sp KDr_4	100	150	100	200	300	12·5	A, Cb, Pn, Su
eijerinckia sp KDr_5	200	600	50	200	500	25	Cb, Cm, Em, Nm, Pn, Su
eijerinckia sp KDr_6	50	100	25	200	300	10	A, Cb, Em, Km, Nm, Pn, Su, Tc
zotobacter sp BNr_1	25	12·5	12·5	25	25	5	A, Cb, Cm, Em, Km, Nm, Pn, Su, Tc
zotobacter sp BNr_3	50	12·5	12·5	50	100	5	Cb, Nm, Pn, Su
zotobacter sp KPr_2	50	100	50	50	50	5	A, Cb, Cm, Em, Km, Nm, Pn, Su, Tc
zotobacter sp KPr_4	100	12·5	12·5	100	25	12·5	Cb, Cm, Em, Km, Pn, Su, Tc
zotobacter sp TPr_3	12·5	12·5	12·5	12·5	12·5	5	A, Cb, Em, Km, Nm, Pn, Su, Tc
acillus sp FSr_5	25	50	12·5	25	25	5	Cm, Em, Nm, Sm
acillus sp PSr_7	50	12·5	12·5	25	25	5	A, Cb, Km, Pn, Su, Tc

Ib, Merbromin; Tm, thimersol; A, ampicillin; Cb, carbenicillin; Cm, chloramphenicol; Em, erythromycin; Km, Kanamycin; Nm, neomycin; 'n, penicillin G; Sm, streptomycin; Su, sulphadiazine; Tc, tetracycline.

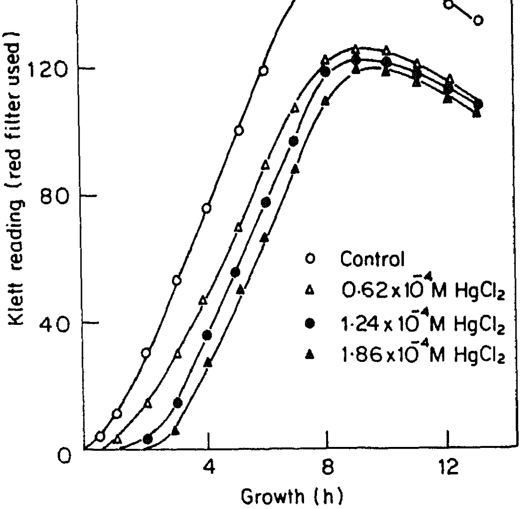

Figure 1. Growth of strain KDr_2 (*Beijerinckia* sp) with or without $HgCl_2$.

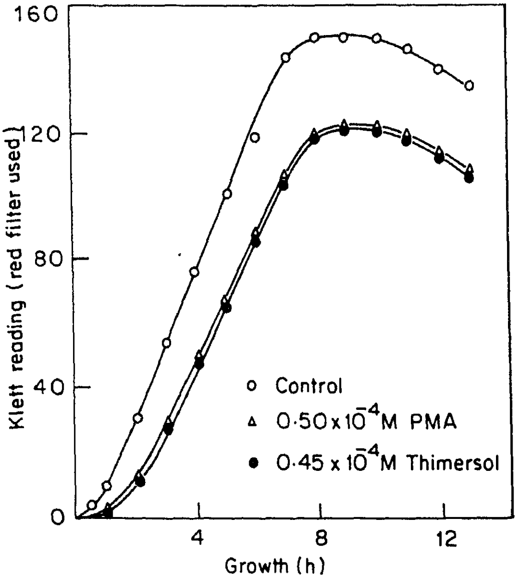

Figure 2. Growth of strain KDr_2 (*Beijerinckia* sp) with phenylmercuric acetate and thimersol.

Azomonas sp MCr_2 and *Bacillus* sp FSr_5 which were weakly Hg-resistant no Hg-reductase enzyme activity was found under our experimental conditions. In all other organisms the Hg-reductase activity was found only when the cells were induced by 10 μM $HgCl_2$. *Beijerinckia* sp KDr_2 showed highest specific activity of the enzyme (0·202 unit). In case of *Beijerinckia* sp KDr_1, *Beijerinckia* sp KDr_5,

Table 2. Volatilization of mercury from $HgCl_2$ containing nutrient broth by mercury-resistant N_2-fixing soil bacteria.

	Control (without organism)	*Azomonas* sp (strain MCr_2)	*Azotobacter* sp (strain KPr_4)	*Azotobacter* sp (strain TDr_1)	*Beijerinckia* sp (strain KDr_2)
Hours of growth	12	12	12	12	12
Wet weight of the cells (g)	—	0·100	0·750	1·000	2·200
Total mercury initially present in 200 ml nutrient broth (mg)	3·333	3·333	3·333	3·333	3·333
Total mercury bound by cells (μg)	—	17·8	3·5	2·4	5·5
Total mercury bound (μg)/g cell mass	—	178·00	4·6	2·4	2·5
Total mercury retained in 200 ml nutrient broth after volatilization (mg)	3·095	2·976	1·071	0·714	0·556
Total mercury lost from 200 ml nutrient broth after volatilization (mg)	0·238	0·3392	2·2585	2·6166	2·7715
Mercury volatilization (%)	7·14	10·18	67·76	78·50	83·15

Table 3. Specific activity of mercury-reductase in mercury-resistant nitrogen-fixing bacteria.

Strain	Concentration of inducer ($HgCl_2$) in μM	Hg-reductase (units/mg protein)
Azotobacter sp TDr_1	0	0
	10	0.066
Azotobacter sp KDr_3	0	0
	10	0·055
Azotobacter sp BNr_1	0	0
	10	0
Azotobacter sp KPr_4	0	0
	10	0·056
Azobacter sp MPr	0	0
	10	0
Azomonas sp MCr_2	0	0
	10	0
Bacillus sp FSr_5	0	0
	10	0
Beijerinckia sp KDr_1	0	0
	10	0·104
Beijerinckia sp KDr_5	0	0
	10	0·105
Beijerinckia sp KDr_2	0	0
	0	0·202

Azotobacter sp TDr_1, *Azotobacter* sp KDr_3 and *Azotobacter* sp KPr_4 the specific activities of Hg-reductase were found to be 0·104, 0·105, 0·066, 0·055 and 0·056 unit respectively.

Hg^{2+} induced oxidation of NADPH at 340 nm by cell-free extract of *Beijerinckia* sp KDr_2 is presented in figure 3. Rate of oxidation of NADPH was much higher in the presence of $HgCl_2$ than on its absence (Komura *et al.*, 1971).

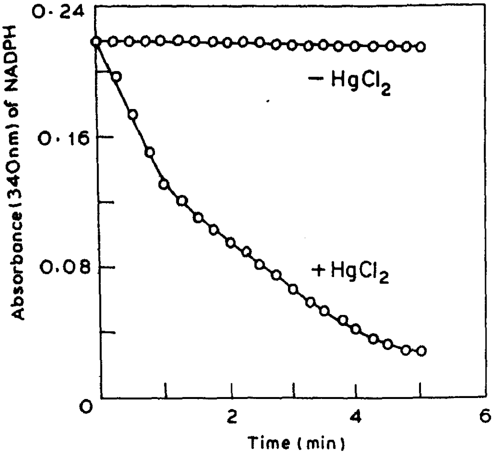

Figure 3. $HgCl_2$ induced oxidation of NADPH by cell-free extract of strain KDr_2 (*Beijerinckia* sp).

Discussion

Out of 24 N_2-fixing bacterial strains resistant to Hg-compounds 16 belonged to the genus *Azotobacter*, 5 to the genus *Beijerinckia*, 2 to the genus *Bacillus* and only one to the genus *Azomonas*. *Azotobacter* sp TDr_1, *Azotobacter* sp TDr_2, *Beijerinckia* sp KDr_1, *Beijerinckia* sp KDr_2, *Azotobacter* sp KDr_3, *Beijerinckia* sp KDr_4, *Beijerinckia* sp KDr_5 and *Beijerinckia* sp KDr_6 displayed resistance to all the mercury compounds tested and may be referred to as 'broad-spectrum' subclass. Among them strains KDr_1, KDr_2 and KDr_5 exhibited very high resistance to all the Hg-compounds and all belonged to the genus *Beijerinckia*. Strains RCr_1, RCr_2 and TPr_3 were highly sensitive to all the Hg-compounds examined and they all belonged to the genus *Azotobacter*. All other bacterial species were referred to as 'narrow-spectrum' subclass. The pattern of mercury and organomercurial resistance properties of the bacteria in 'narrow-spectrum' subclass also differed. Our present work also supports the reports that mercury and organomercurial resistance in bacterial strains is associated with multiple drug resistance properties (Schottel *et al.*, 1974).

The data given in table 1 also suggest that different types (or levels) of detoxifying system may be involved in different level of Hg-resistance observed in these organisms. The data of table 2 indicate that the broad-spectrum Hg-resistant isolates *Beijerinckia* sp KDr_2 and *Azotobacter* sp TDr_1 have high mercury volatilizing capacity of 83·15% and 78·50% respectively in 12 h. *Azotobacter* sp KPr_4, resistant to a number of mercury compounds showed moderate mercury volatilizing capacity *i.e.*, 67·76%. The weakly Hg-resistant organism *Azomonas* sp MCr_2 which belonged to narrow-spectrum subclass showed lowest mercury volatilizing capacity *i.e.*, 10·18% as compared to control (without organism) experiment where 7·14% of total mercury was volatilized.

The amount of mercury bound per gram cell mass of strains KDr_2, TDr_1, KPr_4 were very low (2·5, 2·4 and 4·6 μg respectively). However, in case of weakly mercury-resistant strain MCr_2, the total mercury bound per gram cell mass is very high (178 μg) compared to the organisms mentioned above. The Hg-resistant bacterial cells are reported to volatilize mercury from $HgCl_2$ containing liquid broths and also to bind mercury with the cell constituents (Komura and Izaki, 1971; Hansen *et al.*, 1984; Nakamura *et al.*, 1986; Bhattacharyya and Mandal, 1987). This is evident from our work on *Beijerinckia* sp KDr_2, *Azotobacter* sp TDr_1 and *Azotobacter* sp KPr_4 which have pronounced Hg-reductase activity also rapidly volatilize mercury from the liquid media. The strain KDr_2 having the highest level of Hg-reductase as well as the highest mercury volatilizing capacity, also has very high MIC value against mercury compounds.

Hg-reductase activity in Hg-resistant bacterial cells was always inducible and never constitutive (Summers and Silver, 1978; Mohler *et al.*, 1986). In the 6 N_2-fixing organisms Hg-reductase activity was found only when the cells were induced with 10 μM $HgCl_2$ indicating that Hg-reductase enzyme is also inducible in all these systems. Although the specific activities of Hg-induced oxidation of NADPH of strains KDr_1 and KDr_5 were lower than that of strain KDr_2, yet these activities were higher than those of strains TDr_1 and KDr_3. Interestingly all these 5 strains possessed 'broad-spectrum' Hg-resistant properties. *Azotobacter* sp KPr_4 which is a 'narrow-spectrum' Hg-resistant organism showed a specific activity of 0·056 for Hg-reductase. Other organisms which were tested in our study exhibited no Hg-reductase activity. Our results with N_2-fixing organisms thus support the view that the level of mercury resistance in bacterial cells is associated with inducible Hg-reductase activities in these organisms.

Acknowledgement

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Seasonal reorganization of nitrogen metabolism in an Indian air-breathing teleosts, *Channa punctatus* (Bloch)

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Abstract. *Channa punctatus*, an air-breathing freshwater teleost, mobilizes more protein for its energy requirement during summer and spawning months, as revealed by the data on endogenous nitrogen excretion in the form of ammonia-N, urea-N, free amino acids, creatinine and creatine.

Keywords. Nitrogen metabolism; *Channa punctatus.*

Introduction

Rate of endogenous nitrogen excretion in fishes is an indicator of various environmental and nutritional factors influencing the protein metabolism, and thus gives an insight into the nitrogen economy of fishes (Birkett, 1969; Savitz, 1971). While Gerking (1955) and Savitz (1969) measured the endogenous nitrogen excretion by feeding the fishes a non protein diet (glucose), Kaushik (1980) and Roy and Das (1986) could demonstrate that excretion following a short term starvation (total food deprivation) served as endogenous nitrogen excretion.

A perusal of the available literature reveals no information regarding the investigation on endogenous nitrogen excretion of Indian freshwater teleosts in relation to the annual seasonal variation. Since the air-breathing fishes exhibit a relative dormancy during the summer months (Hora and Pillay, 1962), the present investigation has recorded the seasonal dynamics of excretion of different nitrogenous end products (amino acids, ammonia-N, urea-N, creatinine, creatine, etc.) of common murrel, *Channa punctatus* (Bloch). Thus an attempt has been made for the first time to probe into the seasonal variation in nitrogen metabolism in an Indian air-breathing teleost.

Materials and methods

C. punctatus (30–40 g) were collected from the local market of Santiniketan (23° 39′ N. latitude, 87° 42′ E. longitude, eastern India) in the second week of every month over the season (August 1983–July 1985). The experimental set up for the measurement of endogenous nitrogen excretion was made as per Das and Das (1985). The excretory ammonia-N, urea-N, creatinine and creatine, amino acids and uric acid were quantified according to the methods of Chaney and Marbach (1962), Ashley *et al.* (1968), Bonsnes and Taussky (1945), Moore and Stein (1948) and Blauch and Koch (1939) respectively. These rates were measured at prevailing water temperature of each month. The rates of ammonia-N and urea-N were also measured at constant water temperature of 25°C throughout the annual cycle. The statistical significance of the data was tested through analysis of variance and

Results

Seasonal variation of the rates of ammonia-N, urea-N, creatinine, creatine and amino acids measured at ambient temperature were found to be statistically significant at < 1% level.

The pattern of variation of both the rates of ammonia-N and urea-N were identical (figure 1) with a characteristic peak in the month of September, which was higher than the other two peaks observed in March and May. The levels of excretion during summer months (April–June) were 2-fold more ($P < 0{\cdot}01$) than those of winter months (December–February). When the rates of ammonia-N and urea-N were quantitated at constant temperature of 25°C, the seasonal variation was not found to be statistically significant.

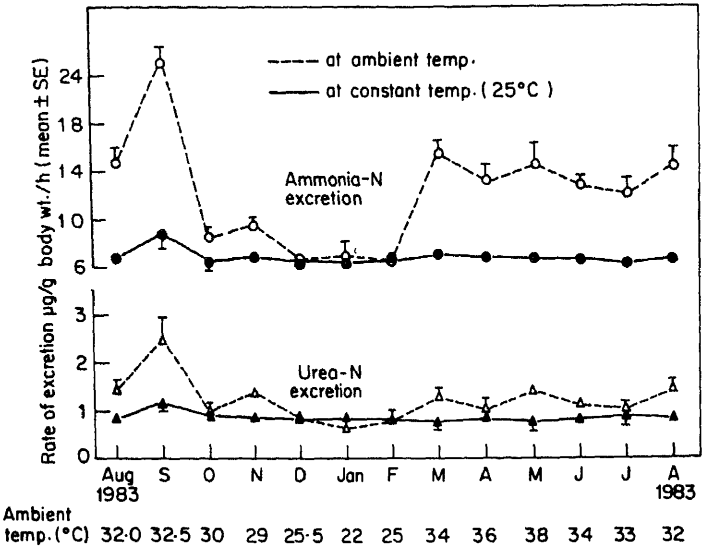

Figure 1. Seasonal variation of the rates of ammonia-N and urea-N excretion of *C. punctatus*, measured at ambient temperature and at constant temperature of 25°C (mean + SE of 6 fishes).

The level of amino acid excretion (measured as ninhydrin positive material) was maximum in January (4·46 µg/g body wt/h) after which the level decreased gradually to a minimum value (0·10 µg/g body wt/h) in September.

The patterns of variation of creatine and creatinine excretion (figure 2) exhibited an inverse relationship. The level of creatine excretion was maximum in June, but the level of creatinine excretion was maximum in August. When the level of creatinine in the excretory medium went up steadily, the level of creatine in the surrounding water declined gradually and *vice-versa*. No detectable uric acid was excreated by *C. punctatus*, as monitored throughout the year.

Discussion

From table 1 it is obvious that *C. punctatus* is 'ammonotelic' like most of the

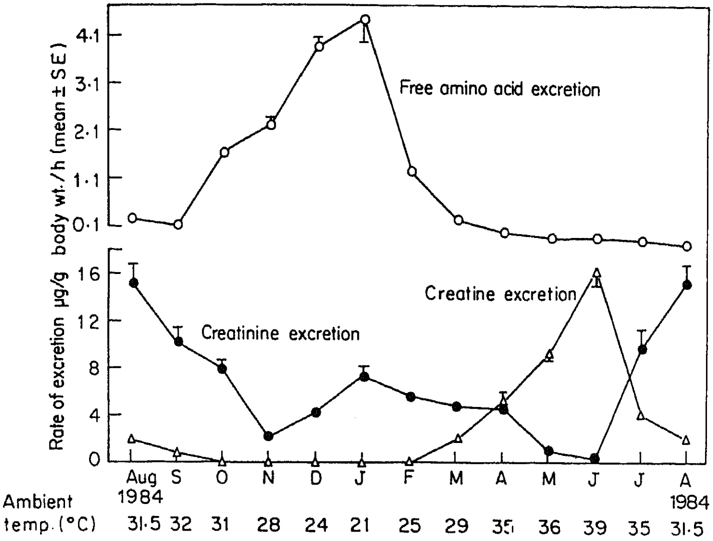

Figure 2. Seasonal variation of the rates of creatinine, creatine and free amino acid excretion of *C. punctatus* measured at ambient temperature (mean + SE of 6 fishes).

Table 1. The excretory rates of different nitrogenous end products of *C. punctatus*.

Nitrogenous end products	Minimal value (µg/g body wt/h)	Maximal value (µg/g body wt/h)
Ammonia-N	6·70	23·33
Urea-N	0·64	2·49
Free amino acids	0·10	4·46
Creatinine	0·004	0·15
Creatine	0·000	0·16

freshwater and marine teleosts (Smith, 1929), and the average ratio of ammonia-N excreted to urea-N excreted ('ammonotelic index') is approximately 9·5 like other air-breathing fishes, while the value of this index is higher in a common carp, *Cirrhina mrigala* (Arya, 1979). In other words, the Indian air-breathing telosts seem to be more 'ureogenic' (Hugins *et al.*, 1969) than the carps. A reduction of the excretory levels of both the ammonia-N and urea-N (figure 1) in the early winter, accompanied by an enhanced excretion of amino acids (figure 2), may be suggestive of a diminution of ammonogenesis and ureogenesis in this fish during the winter. This, however, needs experimental confirmation in future. The increased nitrogenous excretion of ammonia-N, urea-N and even creatine in the summer, to the extent of 2-fold over the levels of winter, may be associated with a greater breakdown of nitrogenus fuels like protein, which is supported by the earlier report of Roy and Das (1987) based on their data on starvation of the fish and of Roy (1988) on the values of ammonia quotient of the fish during summer and winter months.

The depletion of protein in the tissues of *Channa* during summer months, resulting in enhanced nitrogenous excretion, is not associated with an increased activity of the fish in summer. *Channa*, like other Indian air-breathing teleosts, exhibits a relative dormancy in summer. The higher level of nitrogenous excretion of *Cyprinus carpio* during the summer was correlated with the increased metabolic activity of the fish due to the direct effect of increased temperature in summer (Vellas and Serfaty, 1974; Infante, 1974). *Channa*, on the other hand, seems to undergo a seasonal adaptive reorganization in its nitrogen metabolism in relation to the variation of environmental temperature. The season-specific difference in the rates of ammonia-N and urea-N excretion disappear completely, when the rates are measured at a constant temperature.

The rise of the levels of excretory ammonia and urea in September (figure 1) and of creatinine in August (figure 2) may be correlated with the spawning period of this fish, *viz.*, August–September (Mukherjee and Bhattacharya, 1982), when nitrogenous substances like amino acids and protein are likely to be mobilized at a faster rate in order to meet the energy demands for spawning activity. Even the maintenance of higher rates of nitrogenous (ammonia-N and urea-N) excretion throughout the preparatory and pre-spawning phases, *viz.*, March–June (Mukherjee and Bhattacharya, 1982), may be associated with the demands of protein mobilization during the reproductive preparation of the fish. Obviously a 'spent up' fish in the post-spawning period of the early winter exhibits a minimal level of excreted ammonia-N and Urea-N.

Although excretion of free amino acids is well known in several invertebrates (Prosser and Brown, 1961), a perusal of the literatures reveals scanty records of amino acid excretion in freshwater teleosts. While Morii *et al.* (1978) obtained trace amounts of amino acid-N excreted by the estuarine *Periopthalmus* sp. and *Boleopthalmus* sp., their data (0·01–0·08 mg/kg body wt/day) were significantly lower than what could be detected for *C. punctatus* in this investigation during March–September (0·1–0·7 μg/g body wt/h) when the value remains at the minimum level.

The levels of creatinine and creatine excreted by the freshwater teleost *C. punctatus* are more or less comparable to the data of Morii *et al.* (1978) for *Periopthalmus* and *Boleopthalmus* or other marine teleosts (Wood, 1958). An inverse relationship between the rates of excretion of creatinine and creatine (figure 2) is expected, because creatine is the metabolic precursor for creatinine. However, different factors influencing the non-enzymatic spontaneous cyclization of creatine to creatinine are not yet fully explored (Watts and Watts, 1974).

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ctors regulating the transcription of eukaryotic protein coding genes d their mechanism of action—A review

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Abstract. Protein factors play a crucial role in establishing gene-specific and cell-specific regulation of the process of transcription. These include general transcription factors which recognize TATA and CCAAT boxes and which form components of the RNA polymerase II system. Specific transcription factors interact with characteristic promoter elements of individual genes. Some of the examples are SP1, glucocorticoid receptor, GCN4, GAL4 and many others. Transcription factors have a DNA binding domain demarcated from the transcription activation domain. Some factors may have an additional ligand (small molecule) binding domain. Typical structural features such as helix-turn-helix motif, zinc finger and leucine zipper have been recognized in the DNA binding domain of the transcription factors. The acidic domain of the protein factors is involved in the transcription activation process. It appears that activation is the result of the combined action of several regulatory proteins binding at different regions of the promoter. Interaction between proteins bound to DNA but seperated by long stretches of nucleotides is facilitated by DNA bending. Functional specificity as well as diversity are feasible with a limited number of transcription factors through alterations in the architecture of interaction between a group of proteins bound to promoter elements.

Keywords. Transcription factors; promoters; Activation.

roduction

eukaryotes, multiple sequence-specific DNA-protein interactions occurring at tinct upstream regions regulate the initiation of transcription. Promoters of aryotic protein coding genes are characterized by the presence of a TATA box TA$^{A}_{T}$A$^{A}_{T}$) located 20–30 basepairs (bp) upstream of transcription initiation site. TATA box present in eukaryotes is analogous to the Pribnow box (TATAAT) sent in bacterial genes. It specifies the position where RNA synthesis is to begin also determines which of the two DNA strands is to be transcribed (Corden *et* 1980). The sequence G$^{C}_{T}$CAATCT known as the CCAAT box is usually present 90 nucleotides upstream of the transcription initiation site and a family of nscription factors, referred to as the CCAAT binding proteins interact with these uence elements (Chodosh *et al.*, 1988). In addition, several regulatory sequences h as enhancers, silencers etc., located upstream of the TATA box and CCAAT govern the efficiency of transcription. Enhancers have also been detected vnstream of transcription initiation site. Purified RNA polymerase II initiates nscription randomly or at structures such as nicks, free ends etc., and thus

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reviations used: bp, Basepairs; RAPs, RNA polymerase II associated proteins; NFI, nuclear factor I; TF, heat shock transcription factor; GRE, glucocorticoid regulatory element; UAS, upstream vation sequence; HNF, hepatocyte specific nuclear factor; TFIIIA, transcription factor IIIA; C/EBP, AAT box and enhancer binding protein; ATF, activating transcription factor.

control (Lewis and Burgess, 1980). These crude nuclear extracts contain several auxillarly proteins referred to as transcription factors which interact with specific regulatory sequences and bring about accurate and efficient transcription. Several of these factors have been purified and biochemically characterized and are shown to promote specific and accurate initiation of transcription *in vitro*. A large number of transcription factors acting on a variety of eukaryotic promoters in a complex interactive manner have been identified. Some typical examples of transcription factors are listed in table 1. In this brief review, the regulation of RNA polymerase II transcription is discussed with selected examples.

General transcription factors

Factors that bind to common promoter elements such as TFIIA, TFIIB, TFIID, TFIIE and TFIIF isolated from HeLa cell extracts bind to TATA box and promote transcription in cell-free systems. Sopa *et al.* (1986) purified several RNA polymerase II associated proteins (RAPs) utilizing RNA polymerase II affinity columns. These include RAP30, RAP38 and RAP74. RAP30 is a functional component of TFIIF, whereas, RAP38 is required for transcription elongation. SP2 also purified from HeLa cell extracts, is yet another general transcription factor, known to promote transcription from several eukaryotic promoters such as SV40, adenovirus 2 major late promoter, human β-globin and avian sarcoma virus (Dynan and Tjian, 1983). Two factors, A and B are required for accurate initiation of transcription in. *Drosophila* cell extracts. Factor B binds near the TATA box and is compared to TFIIC and TFIID found in HeLa cell extracts (Parker and Topol, 1984a). A protein factor binding specifically to the TATA box of heat shock genes has been identified in *Drosophila*. It is postulated that all the TATA binding functions are governed by a family of proteins, each recognizing a unique sequence surrounding the TATA box core sequence (Wu, 1985).

Several factors binding to the CCAAT box of various eukaryotic promoters have been identified. These include (i) CP1, binding to the CCAAT sequence of human α-globin promoter and adenovirus major late promoter, (ii) CP2, binding to the CCAAT element of γ-fibrinogen promoter and (iii) the nuclear factor I(NFI), regulating transcription and replication in adenovirus. CP1, CP2 and NFI appear to be distinct proteins in that each binds to its own recognition site with an affinity that is several orders of magnitude higher than that with which it binds to the recognition sequences of the other two proteins. These factors, categorized as CCAAT binding proteins, thus show specificity, at the same time perform diverse functions such as regulation of transcription and replication, perhaps through micro heterogeneity in their structure and heteromer formation (Chodosh *et al.*, 1988).

Specific transcription factors

A variety of gene-specific and tissue-specific transcription factors have been identified and only a few examples will be discussed here. The factor SP1 purified from HeLa cell extracts binds specifically the sequence ${}^{G}_{T}\mathrm{GGGCGG}{}^{GGGC}_{TAAT}$ and this

Table 1. Examples of eukaryotic transcription factors*.

Transcription factor	Species/tissue	Target genes
ADR1	Yeast	Alcohol dehydrogenase
AP-1	Human/HeLa	Collagenase
		Metallothionin II
		SV40 enhancer
AP-2	HeLa	Bovine papilloma virus enhancer
	Human/HeLa	Growth hormone
		Metallothionin IIA
		c-myc
		SV40 enhancer
AP-3	HeLa	SV40 enhancer
B factor	*Drosophila*	Actin 5c
		Histone H3
		Histone H4
CCAAT binding factor	Human	hsp70
	Sea urchin	Histone H2B-1
	Mouse	α2 (I) collagen
Estrogen receptor	Chicken	Vitellogenin II
	Human	
	Rat	
GAL4	Yeast	GAL 1–10
		GAL 2
		GAL 7
		GAL 80
GCN4	Yeast	His 3
		His 4
Glucocorticoid receptor	Rat	Glucocorticoid receptor
		Growth hormone
		MMTV LTR
		trp oxygenase
	Rabbit	Uteroglobin
	Chicken	Vitellogenin II
		Lysozyme
	Human	Metallothionin II
HAP 1	Yeast	CYC 1
		CYC 7
HAP 2	Yeast	CYC 1
HSTF	*Drosophila*	hsp70
	Human	hsp70
	Yeast	hsp83
Matα1	Yeast	MFα1
		MFα2
		STE 3
NF-1	Mouse/chicken	MMTV LTR
	Mouse	α2 (I) collagen
	Rat	Albumin
NFκβ	Human B cells	Ig enhancer
Octamer binding factor	HeLa	SV40 enhancer
Progesterone receptor	Chicken	Lysozyme
	Rabbit	MMTV LTR
		Uteroglobin
RC1	Yeast	CYC 1
RC2	Yeast	CYC 1

Table 1. (*Contd.*)

Transcription factor	Species/tissue	Target genes
SP1	HeLa	dhf reductase HIV-1 LTR Metallothionin IIA c-Ha-ras 1 HSV-tk δ1 crystalline U2 Sn RNA
TFIIA	HeLa	AdML
TFIID	HeLa	AdML

*For a complete list of eukaryotic transcription factors and their recognition sequences refer Wingender (1988).

sequence is present in several eukaryotic promoters (Briggs *et al.*, 1986). Kadonaga *et al.* (1987) have shown that amino acids present in the C-terminal region of SP1 factor is responsible for the DNA binding. SP1 is not a tissue-specific factor since it was detected in the nuclear extracts of several tissues.

A heat shock transcription factor (HSTF) binding to the heat shock responsive element (CTnnGAAnnTCnAG) has been identified in *Drosophila*, yeast and man (Widerrecht *et al.*, 1987). HSTF has been purified and shown to stimulate transcription from hsp70 promoter *in vitro* (Parker and Topol, 1984b).

One of the well characterized transcription factors in higher eukaryotes is the glucocorticoid receptor that binds the glucocorticoid regulatory element (GRE) bearing the consensus sequence TGT/CCT. GRE is present in the promoters of several eukaryotic genes such as uteroglobin, metallothionin, chicken lysozyme etc. (Renkawitz *et al.*, 1984a). The binding sites for the progesterone receptor (Jost *et al.*, 1984) and estrogen receptor (Renkawitz *et al.*, 1984b) are also known. In fact, it was reported that a 15 bp sequence mediates both glucocorticoid and progesterone induction of transcription suggesting that these proteins may bind similar sequences (Strahle *et al.*, 1987). The mechanism of activation of transcription by steroid hormone receptors is still not known. According to one view, the receptor is present in the cytosol in complex with a heat shock protein (hsp90) and steroid binding releases the receptor and allows its nuclear localization (Sanchez *et al.*, 1985). Becker *et al.* (1986) argue that the ligand-free receptor itself can bind to target DNA sequences and steroid binding induces a conformational change in the receptor that alters its contacts with DNA. Recently, Godowski *et al.* (1988), have reported that transcriptional enhancement by glucocorticoid receptor requires hormone binding and the unliganded hormone binding region can inhibit all receptor activities other than hormone binding itself. They suggest that the unliganded hormone binding domain forms a complex with hsp90 and the formation of such a complex inhibits receptor function. Hormone binding derepresses receptor activity by altering the conformation of hormone binding domain and disrupting the receptor-hsp90 interactions.

A plethora of factors regulating RNA polymerase II transcription have been identified in yeast. These include GAL4, GCN4, HAP1, HAP2, MATα2 and ADR1 gene products. The GAL4 protein regulates the transcription of GAL1 gene by binding to an upstream activation sequence (UAS) called UAS_G. The first 74 Nd residues 148–196 and 768–881 are involved in transcription activation (Ma and ıshne, 1987a).

Ƭhe GCN4 protein promotes transcription of several co-regulated genes in ponse to amino acid starvation (general amino acid control). GCN4 protein ıds a 9 bp palindrome, ATGAC/GTCAT, which is also recognized by the mmalian transcription factor AP1 as well as the jun oncoprotein (Rausher *et al.*, 38). The DNA binding domain of GCN4 protein lies in the 60 C-terminal amino d residues. The C-terminus also has a 19 amino acid long acidic domain that is plicated in transcription activation function (Hope and Struhl, 1986).

Ƭhe iso-1-cytochrome C gene (CYC1) of yeast contains two UASs known as ıS1 and UAS2 which interact with proteins coded by HAP1, HAP2 and HAP3 ı (Guarente and Hoar, 1984). HAP1 product binds at UAS1, whereas UAS2 is ivated by the combined action of HAP2 and HAP3 proteins. HAP1 protein also ıds the UAS of CYC7 gene with equal affinity. However, there is no sequence nology between UAS1 of CYC1 gene and the UAS of CYC7 gene (Pfeifer *et al.*, 37). Thus HAP1 protein is a rare example of a transcription factor binding two related sequences. Other transcription factors identified in yeast include the ıTα2 protein (Johnson and Herskowitz, 1985) and the ADR1 gene product ırtshore *et al.*, 1986). The latter is required for the transcriptional activation of ohol dehydrogenase gene.

Several transcriptional factors expressed in selective tissues have been identified. ıiver specific transcription factor referred to as NFI-L, binding to a sequence ıilar to that recognized by NFI (TGGCA) has recently been identified (Paonessa *al.*, 1988). Similarly, Courtois *et al.* (1988), purified a 88 kDa protein binding to α_1-antitrypsin gene promoter and this is identified as hepatocyte specific nuclear tor (HNF1). A pitutary-specific factor called Pit-1, regulating the transcription of olactin and growth hormone genes in the lactotrophs and somatotrophs of ıtary gland has been reported (Nelson *et al.*, 1988). An erythroid-specific factor led ERYF-1, binding to the sequence AGATAA of chicken globin genes was ıwn to play a prominent role in the initial establishment of erythroid cell lineage ans *et al.*, 1988). Other tissue-specific factors include the octamer binding protein mmunoglobulin genes expressed in B cells and that expressed in sea urchin testis rberis *et al.*, 1987). Several factors claimed to be tissue-specific, were later found induce transcription in other tissues as well. For example, the factor NF-κβ, ding to Igκ-enhancer and expressed only in κ-producing cells was later shown to induced by phorbol esters in HeLa cells as well (Sen and Baltimore, 1986).

ucture of the DNA binding domain of transcription activators

spite several unique characteristics of their own, transcription factors share some nmon structural features so that certain generalizations can be made. Majority of se factors are sequence-specific DNA binding proteins and contain two major ctional domains, namely, the DNA binding domain and the transcription ivation domain. Proteins like steroid hormone receptors possess an additional nain for ligand binding. In addition, several of these proteins interact with other oteins of the transcription machinery through their carboxy terminii. Many of the nscription factors show considerable homology in their DNA binding domains and studies so far have led to the identification of 3 major DNA binding motifs, namely, the helix-turn-helix motif, the zinc finger motif and the leucine zipper.

Helix-turn-helix motif

This motif was first identified in the DNA binding domain of prokaryotic regulatory proteins such as λ-repressor, cro protein, *Escherichia coli* Gal repressor, Lac repressor and Trp repressor (Pabo and Sauer, 1984). A similar motif was later identified in the yeast MATα2 protein and certain homeotic products of *Drosophila* (Sheperd *et al.*, 1984). This motif is characterized by the presence of two α-helices, one of which called the helix3, lies within the major groove of DNA while the other lies across. Helix3 plays a major role in DNA recognition and mutation of amino acids in helix3 leads to weak promoter recognition. Amino acids that form the hinge region between the two α-helices are highly conserved among all these proteins. The helix-turn-helix motif lies in the amino terminus while the carboxy terminal amino acids provide sites for the dimerization of two monomers such that the axis of 2-fold symmetry of the dimer is coincident with that of DNA. These proteins dock with operators in the B-form. Hydrogen bonds and van der Waals forces are involved in the interactions between the amino acid side chains and edges of bases in the major groove of DNA. These, together with the backbone and electrostatic interactions stabilize DNA-protein complexes (Ptashne *et al.*, 1982) (figure 1).

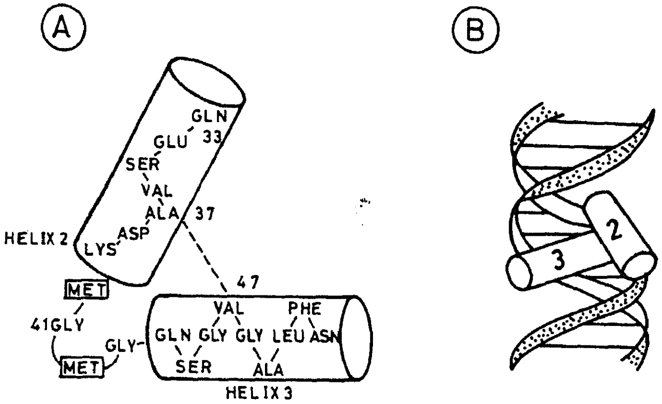

Figure 1. (**A**) The helix-turn-helix motif of λ-repressor. Amino acids 33(GLN), 37(ALA), 41(GLY) and 47(VAL) are highly conserved among all the prokaryotic repressor proteins. In addition, glycine at position 41 is usually flanked by hydrophobic amino acids. The angle between the helix2 and helix3 is controlled by the contact between the side chains of alanine at position 37 and valine at position 47. (**B**) Interaction of λ-repressor with the operator DNA.

Zinc finger motif

The zinc finger motif was first identified in the DNA binding domain of transcription factor IIIA (TFIIIA) which activates transcription from promoters recognized by product of yeast, the gag gene product of retroviruses, E1A gene product of adenovirus, large T antigen of papova viruses, the SP1 factor and the product of *Drosophila* developmental regulatory loci like kruppel and serendipity (Berg, 1986; Rosenber, 1986). The sex determining region of human Y chromosome was shown to encode a finger protein (Page *et al.*, 1987).

TFIIIA is a 40 kDa polypeptide with a 30 kDa aminoterminal domain retaining DNA binding activity and a 10 kDa carboxy terminal domain involved in transcription activation (Smith, 1984). Zinc (7–11 atoms) are complexed with each TFIIIA molecule. The DNA binding domain of TFIIIA consists of 9 homologous subunits termed zinc fingers. Each zinc finger is composed of 30 amino acids and contain the sequence of the form Cys-X$_{2-5}$-Cys-X$_{12}$-His-X$_{2-3}$-His, where X may be any amino acid. Each of the 30 amino acid domains of TFIIIA forms a loop or finger of 12 amino acids, the base of which is formed by Zn^{2+} bound by Cys and His residues (figure 2). The amino acids in the loop bind DNA with each finger interacting with one half turn of the helix (Rhodes and Klug, 1986). Analysis of the crystal structure of a double stranded nonadeoxynucleotide that corresponds to the tightest binding part of the sequence recognized by TFIIIA revealed that it has an A-type rather than a B-type conformation (McCall *et al.*, 1986). TFIIIA molecule binds to the internal control region of the 5S rRNA gene between the nucleotides +40 and +90 and this interaction is stabilized by the sequential binding of two other protein factors called B and C. Each of the 9 finger domains of TFIIIA interacts with about half a DNA period and thus the 9 domains cover the entire internal control region. An advantage of the multiple fingers is that the stable transcriptional complex, once formed, can sustain several rounds of transcription, during which process the factor remains bound to the gene. As the RNA polymerase III passes through the gene, the multifingered protein releases the fingers bound ahead of the progressing polymerase but remains bound to the DNA by its remaining fingers (Miller *et al.*, 1985).

When genomic libraries of different animals were screened with a probe coding

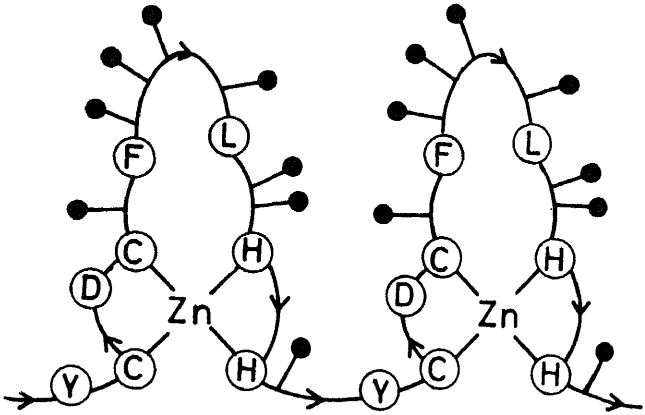

Figure 2. The zinc finger motif of Xenopus TFIIIA. Only two of the 9 repetitive motifs are shown. (●), Most probable DNA binding side chains. F, phenyl alanine; L, leucine; C, cysteine; H, histidine; D, aspartic acid; Y, tyrosine.

for the finger domain, several copies of homologous DNA sequences were detected suggesting that several finger proteins exist in eukaryotes and these are well conserved during evolution (Schutz *et al.*, 1986).

Leucine zipper

Landschulz *et al.* (1988) identified a new motif termed leucine zipper in the CCAAT box and enhancer binding protein (C/EBP) of rat liver nuclei. A computer search revealed that a similar motif is present in several other DNA binding proteins such as mouse c-myc, human N-myc, human L-myc, v-jun, v-fos and GCN4. The region of homology is characterized by a periodic repetition of leucine residues at every seventh position over a distance covering 8 helical turns. The leucine repeats in these proteins can form an α-helix with the leucine side chains projecting out from the helix at regular intervals (figure 3A). It was proposed that the leucine side chains from one protein molecule interdigitate with those on the other, forming a zipper that holds the two molecules together and this dimerization facilitates appropriate positioning of amino acid side chains of these proteins into the grooves of the double helical DNA (figure 3B). Thus the leucine zipper is not directly involved in DNA binding but aligns the interacting protein molecules alongside the DNA such that atomic interactions can take place between the amino acid side chains and the bp of DNA.

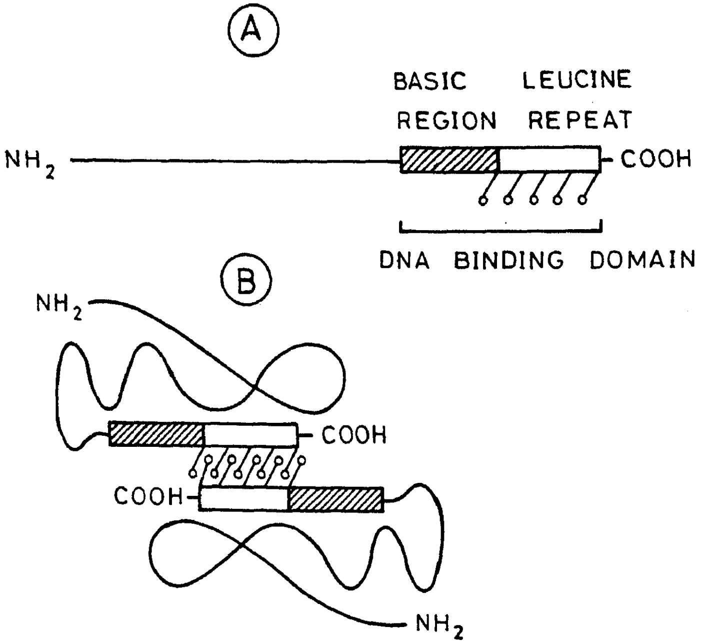

Figure 3. Leucine zipper of the C/EBP. (**A**) Location of the leucine repeat within the C/EBP polypeptide. The DNA binding domain lies beyond the leucine zipper domain towards the aminoterminus of the protein (striped region). (**B**) A hypothetical model of the C/EBP dimer formed by the interdigitation of the leucine zipper domains in an antiparallel conformation.

chanism of transcription activation

e of acidic amino acids in transcription activation

lysis of the transcription activation domain of certain transcription factors ealed that a group of acidic amino acids conferring a net negative charge may an important role in activation of transcription. It was observed that the nsactivation domain of GAL4 protein is composed of several acidic amino acids mutational analysis confirmed that the negative charge is essential for transcrip- activation function (Gill and Ptashne, 1987). The activation domain of GCN4 ein also bears an acidic character but the amino acid sequence in this region s no homology to that of GAL4 protein (Ma and Ptashne, 1987b). Although ic amino acids seem to impart transcription activation function to these eins, there is poor correlation between the strength of activation and the ber of acidic amino acid residues. Single amino acid changes reduce GCN4 vation potential without much change in acidic character. Giniger and Ptashne 7) constructed a gene encoding a 15 amino acid long polypeptide that is ble of forming an α-helix, one surface of which consists of negatively charged no acids, while the other bears hydrophobic amino acids. When attached to a A binding domain, this peptide was found to activate transcription in yeast. s higher level of structural determinants such as an amphipathic helix with ged amino acids on one face, may be more important than the actual amino sequence, in transcription activation.

he transcription activation domain of gene regulatory proteins need not always cidic as shown in the case of glucocorticoid receptor, which consists of two well acterized enhancement domains, enh1 and enh2. The enh2 domain is very lar to the activation domain of GAL4 protein in containing several acidic no acids and the distribution of negative charge is also similar between the two eins. However, the enh1 domain is positively charged, containing 6 acidic and asic amino acid residues between amino acids 440 and 525 and it can function pendantly of enh2 domain. It is suggested that these enhancement domains it display distinct activities in different cells or they might operate by entirely rent mechanisms (Godowski *et al.*, 1988).

rid gene regulatory proteins

of the current approaches for the understanding of the mechanism of trans- ion activation involves construction of hybrid regulatory proteins. It was n that a chimeric protein consisting of the DNA binding domain of estrogen otor and transcription activation domain of either GCN4 or GAL4 protein can ate a promoter containing estrogen responsive element (Webster *et al.*, 1988). larly, a hybrid protein containing the DNA binding domain of *E. coli* lexA essor and the trans-activation domain of GAL4 protein was able to activate cription from a promoter containing lexA operator. In yet another study, the binding domain of progesterone receptor was replaced by the corresponding n of glucocorticoid receptor and this hybrid protein specifically binds the ocorticoid regulatory element and leads to progesterone dependant activation of ologous genes (Green and Chambon, 1987). Recently, Hollis *et al.* (1988), constructed a hybrid phage 434 repressor in which the DNA recognition helix was replaced by the corresponding region of phage P22 repressor. This chimeric protein binds specifically the P22 operator. Further, a dimer comprising of the 434 repressor monomer and the chimeric 434-P22 repressor monomer specifically recognizes a chimeric operator that lacks the usual 2-fold symmetry, suggesting that combination of two DNA binding proteins may form a novel protein that recognizes a new DNA sequence.

Protein-protein interactions

The binding sites of gene regulatory proteins often lie several bp upstream of transcription start site. Thus arises the question as to how do these proteins, binding far upstream regions regulate transcription? A glimpse at the various eukaryotic genes reveals that transcription activation, often, is the result of the combined action of several regulatory proteins binding at different regions of the promoter. It is suggested that these proteins interact with one another leading to the formation of a DNA loop thereby bringing the upstream sites adjascent to the transcription start site. The evidences for such protein-protein interactions are, infact, overwhelming. For example, the carboxy terminal amino acids of TFIIIA are essential for the correct initiation of transcription and it is suggested that this region is involved in the binding of other components of the transcription machinery (Harrison, 1986). Similarly, the promoters of heat shock genes contain multiple copies of heat shock elements and it is proposed that the HSTF binds at these sites in a cooperative fashion and the binding of the first HSTF molecule causes a bend in DNA that facilitates the binding of the second HSTF molecule, whose binding results in additional conformation changes in the contacts of DNA to HSTF, triggering transcription (Shuey and Parker, 1986). In amphibian oocytes, heat shock element as well as the CCAAT box are essential for the efficient binding of HSTF to hsp70 promoter suggesting that the CCAAT box binding protein may interact with HSTF, thereby enhancing the affinity of the latter for the promoter (Beinz, 1986). The recognition sequences of SP1 factor are often found near the binding sites of other transcription factors such as AP1 or NF1 and it is believed that these factors may act in conjunction with one another and modulate transcription (Kadonaga *et al.*, 1987). Similar observations have been made in prokaryotes as well. For example, it was shown in the case of λ-repressor, which binds at the right operator (O_R) of the λ-phage promoting the transcription of genes involved in lysogeny, that mutations in the amino terminal domain seriously disrupt the transcription activation function but the DNA binding function remains unaffected. It was suggested that the amino terminus represents the positive control domain and the repressor interacts with RNA polymerase through its amino terminal amino acids (Ptashne, 1986). It has recently been reported that the yeast GAL4 protein can activate transcription synergistically in conjunction with several mammalian transcription factors such as activating transcription factor (ATF), upstream stimulating factor and glucocorticoid receptor (Kakidani and Ptashne, 1988; Webster *et al.*, 1988). It is believed that the interaction between any two transcription activators may be mediated by a common target protein such as TFIID, a TATA binding protein. Infact, it has been shown by Horikoshi *et al.* (1988), that ATF binds to multiple upstream elements of adenovirus E4 promoter l alters the promoter interactions of TFIID and these interactions in turn litate promoter recognition by RNA polymerase II and the subsequent nation of pre initiation complex.

t is to be mentioned here that motifs such as the leucine zipper play a very portant role in facilitating protein-protein interactions among different nscription activators. For example, it has been shown recently that the leucine per stabilizes the interaction between fos and jun proteins and the fos-jun eromer binds to DNA more tightly than either protein alone (Kouzarides and , 1988; Sassone-corsi *et al.*, 1988). Thus, different transcription activators may eract with one another through motifs such as the leucine zipper and such eractions may lead to several combinations of dimers. A large number of such eromers may indeed be involved in the regulation of transcription of eukaryotic e families.

elching

l and Ptashne (1988) observed that the yeast GAL4 protein, when expressed at n levels, inhibits transcription of certain genes that lack GAL4 binding sites. This ibitory effect, known as squelching, is independant of DNA binding domain, but olves the transcription activation domain. A GAL4 protein, lacking the DNA ding domain inhibits, whereas, a mutant lacking the activator domain does not ibit. GAL4 derivatives possessing stronger activating domains inhibit more ciently. It is suggested that squelching may be the result of the interaction ween the activating domain of transcription factor and a target protein such as IID. When the transcription factor binds to DNA, its activating domain is ught into the immediate vicinity of the gene, where it interacts with a specific get protein leading to the formation of an active transcription complex. The target tein is present in limiting amounts and an activator protein may interact with former even without binding to DNA and as a result, the transcription is cked because of the nonavailability of the target protein. Thus, overproduction ι transcription activator would repress transcription from promoters lacking the vator binding sites and at very high concentrations, promoters bearing the ding sites would also be inhibited. These results suggest that the rate of nscription depends on the concentration of activators in the cell and the vator concentration should be regulated such that the activating domains are osed only when required. The following examples indicate that such a regulation y indeed be operating *in vivo*. In yeast, the negative regulatory protein GAL80 ds to the activating domain of GAL4 in the absence of galactose, thereby cking transcription. In higher eukaryotes, steroid hormone receptors are nplexed with hsp90 in the absence of steroid hormone. Several transcription ors are turned over rapidly, whereas in others, the activating domain is ctional only when phosphorylated and regulation is effected at the level of osphorylation (Ptashne, 1988).

transcription. Prototype chemicals such as phenobarbitone and 3-methylcholanthrene enhance the transcription of cytochrome P-450 b/e and c/d genes by 20–50-fold in the whole animal after a single injection (Ashwanikumar and Padmanaban, 1980; Ravishankar and Padmanaban, 1985). An interesting finding has been that heme, the prosthetic group of cytochrome P-450, is a positive modulator of the transcription of this gene (Ravishankar and Padmanaban, 1983; Sathyabhama *et al.*, 1986; Bhat and Padmanaban, 1988). Studies also reveal that cycloheximide inhibits phenobarbitone or 3-methylcholanthrene mediated activation of the transcription of the respective P-450 genes implicating the possible involvement of a drug-induced transcription factor (Bhat *et al.*, 1987). In support of this, nitrocellulose filter binding, gel retardation, foot print assays and south western blot analysis of the interaction between the upstream region of the P-450 b/e gene (Rangarajan *et al.*, 1987) and nuclear extracts, reveal drug- and heme-modulated binding of a transcription factor(s) to the upstream sites. A major protein involved has a molecular weight of 85 kDa. The binding of the transcription factor(s) to the upstream region correlates very well with the pattern of activation of transcription under different conditions of treatment (Rangarajan and Padmanaban, 1989). The system offers excellent scope to dissect the various processes associated with the phenomenon of transcription.

Prospects

Eukaryotic transcription, once considered an impenetrable black box, is now amenable for detailed analysis and significant information has already been accumulated. The generation of cell-free transcription systems responding to tissue and gene specific factors will help in understanding the process of selectivity in transcription activation. Negative interactions are as important as the positive ones, since the process of inactivation of genes and reasons for their silence need to be understood. The involvement of higher order structures of the genome in transcription regulation is still an open question. The main problem is to seggregate the cause from effect. Despite the complexity of chromatin structure, it is interesting to note that transcriptional activation state in a variety of systems is reflected in the functional consequence of the interaction between naked DNA and the transcription factors involved. Another interesting fact is the possible functional diversity of the transcription factors, which while manifesting specificity in one plan of architecture arising out of interaction with a set of proteins may show a different specificity in another combination or even assume the function of a replication factor. Finally, the regulatory potential of the different facets of transcription, namely, initiation, elongation and termination have to be understood and integrated.

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racellular phosphate solubilization by the cyanobacterium *Anabaena* M310

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Abstract. Endogenous polyphosphate depleted *Anabaena* ARM310, solubilized extracellular tricalcium phosphate through increased phosphatase activity.

Keywords. Extracellular phosphate solubilization; involvement of phosphatases.

duction

bilization of fixed soil phosphates by microorganisms for greater assimilation
lants is of practical importance. Different groups of phosphate solubilizing
oorganisms, particularly bacteria and fungi, have been reported to render
ganic phosphatic compounds soluble (Bardiya and Gaur, 1972; Wani *et al.*,
). Gaur (1988) in his recent review on phosphate solubilizing microorganisms
implicated the organic acids produced by fungi and bacteria in phosphate
bilization.

anobacteria are increasingly used as bio-fertilizers for rice (Venkataraman,
). An efficient nitrogen fixing and phosphate solubilizing strain of
obacterium will be very useful for the cultivation of rice where bound
phates remain unavailable. In the present investigation, the ability of *Anabaena*
1310, a heterocystous nitrogen fixing cyanobacterium, to solubilize extracellular
uble tricalcium phosphate using phosphatases was studied.

erials and methods

obacterial strain

aena ARM310 was obtained from the Division of Microbiology, Indian
cultural Research Institute, New Delhi.

ire medium and growth conditions

aena ARM310 was maintained in BG11 medium (Rippka *et al.*, 1979). The pH
ne medium was adjusted to 7·2 using 0·1 N NaOH before autoclaving.
ures were maintained as batch cultures at 26 ± 1°C with a light intensity of
–2000 lux, and grown in a light/dark cycle of 12/12 h.

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vhom all correspondence should be addressed.

Phosphate starvation

The filaments of *Anabaena* ARM310 were subcultured in BG11 medium containing combined nitrogen but without dipotassium hydrogen phosphate. However, potassium chloride was added to maintain the availability of potassium in the medium. Twenty to twenty five day old cultures were used as inocula for experiments.

Insoluble phosphate used in the experiments

Tricalcium phosphate [$Ca_3(PO_4)_2$], was added at a concentration of 1 mg/ml (w/v) of medium.

Growth

Pre-weighed phosphate starved cells were inoculated and subjected to the following treatments.

(A) $CN^+ SP^-$ – BG11 medium containing combined nitrogen but without dipotassium hydrogen phosphate.
(B) $CN^+ SP^+$ – BG11 medium containing both combined nitrogen and dipotassium hydrogen phosphate.
(C) $CN^+ ISP^+$ – BG11 medium containing both combined nitrogen and insoluble tricalcium phosphate.
(D) $CN^- SP^-$ – BG11 medium without combined nitrogen and dipotassium hydrogen phosphate.
(E) $CN^- SP^+$ – BG11 medium without combined nitrogen but with dipotassium hydrogen phosphate.
(F) $CN^- ISP^+$ – BG11 medium without combined nitrogen but with insoluble tricalcium phosphate.

All the treatments were carried out in duplicates. The end point growth was recorded after 25 days of culture in all the treatments.

Estimation of chlorophyll a

Chlorophyll *a* was estimated by the method of Mackiney (1941). Harvested cells from each treatment were suspended in 1 ml of distilled water. Then 4 ml of methanol was added and the tubes were incubated in a waterbath at 60°C for 30 min. The tubes were then centrifuged for 10 min and the supernatant was used to read the absorbance at 663 nm.

pH estimation

Phosphate solubilization and assay of phosphatase

Phosphate solubilization was studied in terms of extracellular phosphatase activity in the culture filtrates. The activity of phosphatase was assayed on 10th, 15th and 20th day of growth. Alkaline phosphatase activity in the culture filtrates was assayed by suitably modifying the procedure of Freeland (1985). The alkaline phosphatase assay mixture contained 1·5 ml of 0·4 M Tris-HCl buffer (pH 8·5), 0·1 ml of 16·6 mM phenolphthalein diphosphate and 1·4 ml of culture filtrate from the inoculated flasks, in a total volume of 3 ml. Tubes containing the assay mixture were kept for 30 min in dark at room temperature. After 30 min, the reaction was terminated by adding 0·1 ml of 1 M dipotassium hydrogen phosphate. The absorbance of the supernatant, which turned pink due to the release of phenolphthalein, was measured at 540 nm. Uninoculated culture medium in a total volume of 3 ml of the assay mixture was used as blank. The amount of phenolphthalein released was measured from the phenolphthalein standard graph.

Protein in the culture filtrate was estimated following the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

The alkalaine phosphatase activity is expressed as μg of phenolphthalein liberated per μg protein per min.

Acetylene reduction assay

Phosphate starved cells of *Anabaena* ARM310 were inoculated in flasks containing the treatments E and F. Nitrogenase activity was determined following the acetylene reduction technique of Stewart *et al.* (1968). Ten and fifteen day old algal samples were taken in 1 ml of medium in serum bottles (6 ml capacity) separately. The bottles were closed with a subaseal rubber stopper. After 6 h of stabilization in the medium, 1 ml of pure acetylene was injected into each bottle. The algal samples in serum bottles were incubated again for 6 h under 1500 lux light intensity. The reaction was terminated by injecting 0·2 ml of 20% trichloroacetic acid. One ml of gas sample from each serum bottle was analysed using 5830 A Hewlett Packard microprocessor attached gas chromatograph fitted with a flame ionisation detector and a porapak column.

The algal pellet was digested in 5 ml of 0·5 N NaOH to estimate protein following Lowry *et al.* (1951). Nitrogenase activity is expressed in terms of nmol of ethylene formed per mg protein per hour.

Results and discussion

Growth

The growth of *Anabaena* ARM310 was measured in terms of chlorophyll *a* content (figure 1). In the presence of phosphates, soluble or insoluble, growth was greater than in the absence of phosphates. The growth observed in the present study indicates that the organism could utilise extracellular insoluble phosphates under both CN^+ and CN^- conditions. A number of other cyanobacteria, such as *Anabaena*, *Nostoc*, *Tolypothrix*, *Aulosira* and *Anacystis*, have been shown to solubilize extracellular insoluble phosphates (Bose *et al.*, 1971).

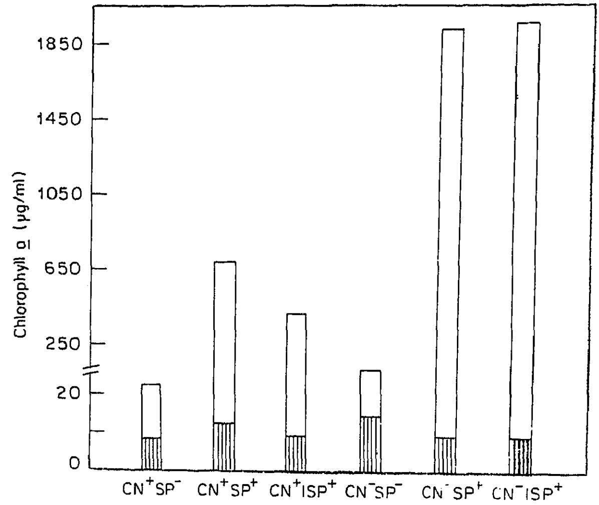

Figure 1. Growth of *Anabaena* ARM310. (▥), Chlorophyll *a* content of the initial inoculum; (□), chlorophyll *a* content of cells at the end of the growth period.

Phosphate solubilization

The pH of the culture filtrate containing different phosphate sources with and without combined nitrogen showed marginal increase over the respective uninoculated control (table 1). Earlier reports in bacteria indicate acid hydrolysis by organic acids as a possible mechanism of phosphate solubilization (Subba Rao, 1982). Sperber (1958) reported that acids secreted by the organisms lowered the pH

Table 1. pH of the culture filtrates of *Anabaena* ARM310.

Treatment	Sample	Zero time	Days after inoculation 5	10	15	20	25
A CN^+ SP^-	UC	7·2	7·2	7·2	7·2	7·2	7·2
	Exp.	7·2	7·5	8·0	7·7	7·6	8·0
B CN^+ SP^+	UC	7·2	7·2	7·2	7·2	7·2	7·2
	Exp.	7·2	7·3	7·8	7·8	7·7	8·1
C CN^+ ISP^+	UC	7·2	7·2	7·2	7·2	7·2	7·2
	Exp.	7·2	7·3	7·3	7·5	7·7	7·8
D CN^- SP^-	UC	7·2	7·2	7·2	7·2	7·2	7·2
	Exp.	7·2	7·3	7·6	7·8	7·7	7·5
E CN^- SP^+	UC	7·2	7·2	7·2	7·2	7·2	7·2
	Exp.	7·2	7·3	7·8	7·5	7·7	7·8
F CN^- ISP^+	UC	7·2	7·2	7·2	7·2	7·2	7·2
	Exp.	7·2	7·3	7·3	7·5	7·4	7·5

Data represent average of two estimations.

UC, Uninoculated control. Exp., Experimental.

CN^+, Medium with combined nitrogen; CN^-, medium without combined nitrogen; ISP^+, medium with tricalcium phosphate; SP^+, medium with dipotassium hydrogen phosphate; SP^-, medium without dipotassium hydrogen phosphate.

the medium and thereby dissolved the bound phosphates. However, in the esent study this does not seem to happen since the pH of the culture filtrate never opped to·the acidic range.

Under conditions of phosphate starvation cyanobacteria are known to show creased levels of intracellular and cell surface alkaline phosphatase activity to lubilize polyphosphates (Healey, 1982). There was a shift in the pH towards calinity in all the inoculated treatments while in the uninoculated treatments the I remained unchanged. This observation prompted us to investigate the volvement of alkaline phosphatases in solubilization of extracellular phosphates.

Under conditions of soluble phosphate depletion and insoluble phosphate dition (figure 2A, C), alkaline phosphatase activity was observed from the 10th y, which increased on the 15th and 20th day. However, in the presence of soluble osphates the activity was detected only from the 15th day (figure 2B). This lends pport to the argument that alkaline phosphatases are induced and secreted on osphate starvation/depletion and that these are involved in the solubilization of calcium phosphate.

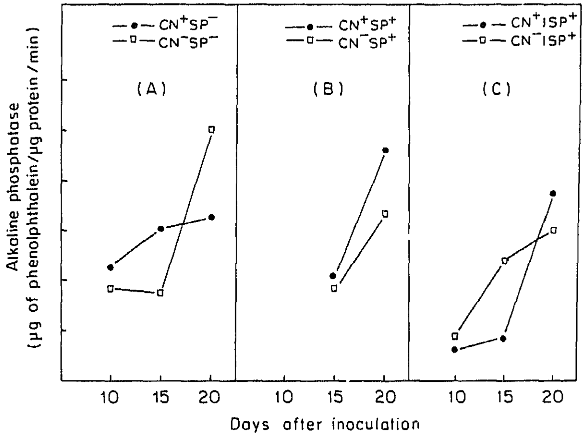

Figure 2. Alkaline phosphatase activity in the culture filtrate of *Anabaena* ARM310.

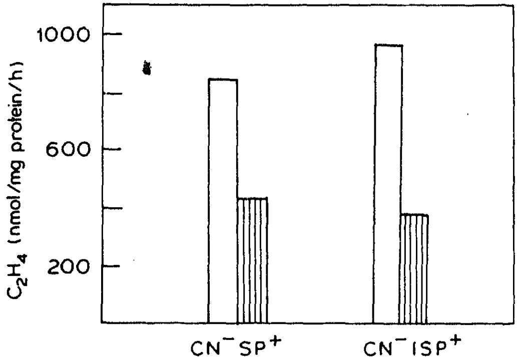

Figure 3. Acetylene reduction assay of *Anabaena* ARM310. Nitrogenase activity of the cells on 10th (□) and 15th (▥) day.

Acetylene reduction activity

There was no significant difference in the acetylene reduction activity of the cells incubated in treatments E and F (figure 3). The cyanobacterium *Anabaena* ARM310 seems to be an ideal choice for the preparation of microbial inoculant because of its ability to grow well and fix atmospheric nitrogen in the absence of combined nitrogen and soluble phosphates.

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Immunochemical identification of mung bean actin like protein and its cellular involvement during germination

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Abstract. Actin like protein, extracted and purified from *Vigna radiata* (mung bean) seedling, has been found to give positive enzyme-linked immunosorbent assay with mouse monoclonal antiactin antibody.

In vivo studies show that cytochalasin B at sublethal dose inhibits the chromosomal movement at metaphase stage during germination. From *in vitro* studies it is found that the actin like protein isolated from mung bean seedling has a cytochalasin B binding property with a Kd value $1{\cdot}2 \times 10^{-5}$ M. From these two specific observations it appears probable that the biological function of mung bean actin like protein is to take part in cell division process directly or indirectly during the time of seedling development.

Keywords. *Vigna radiata*; ELISA; cytochalasin B.

Introduction

Actin, which was once thought to be localized exclusively in muscle cells, has now been found to be associated with many types of non muscle motilities as for example ameboid movement, cytokinesis and cytoplasmic streaming (Forer, 1974; Pollard and Weighing, 1974; Sanger, 1975). Sanger in 1975 observed the presence of actin in the mitotic spindle of rat Kangaroo cells with the help of fluorescent-labelled heavy mero-myosin (HMM). According to him, the actin in the spindle is confined to the fibers, that connect the chromosome with the centriolar region. Moreover the notable fibrilar structure associated with cytokinesis is the contractile ring, whose short lived presence as a band or ring like array of microfilaments was observed under electron microscope (Schroeder, 1972). Their resemblance to actin was demonstrated by complexing with HMM and therefore it was concluded that contractile ring contains an actin like component (Perry *et al.*, 1971). Furthermore, cytochalasin B brings about the disruption of contractile ring microfilaments (Schmiedel and Schnepf, 1980). The specificity of these results strongly suggests that microfilaments of the contractile ring are closely related to muscle actin and hence have been considered as a mechanochemical organelle that cause cell cleavage.

Antibodies against actin have been experimentally produced in animals in recent years (Fagraeus and Norberg, 1978). It has been reported in case of higher plants, for example soybean (Metcalf III *et al.*, 1980) and wheat germ (Ilker *et al.*, 1979), that the actin like protein cross reacts with anti-actin antibody produced against muscle actin. A major difficulty in the immunochemical approach to actin is the poor immunogenecity of this antigen. It has not been possible to raise antibodies against mung bean actin like protein. A quantitative study of the antigenic reactivity of mung bean actin like protein by following enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody raised against mouse actin has been presented here.

In mung bean seedling, there is no apparent contractile event related to movement where the actin may participate actively. Hence the question may arise as to which particular cellular process involves the function of mung bean seedling actin. The fact that the mung bean seedling is growing fast during germination indicates that the actin like element present in this system may have some role during cell division. The present study describes the work which shows the antigenic reactivity of mung bean actin like protein and also its role during cell division.

Materials and methods

Chemicals and radiochemicals

Conjugate of peroxidase (from horse radish) and protein A (from *Staphylococcus aureus*), 2,2′-azino-di (3-ethylbenzthazoline) sulphonate (ABTS), a tritiated cytochalasin B [4(n)–^{3}H] and mouse monoclonal anti-actin antibody were obtained from Radiochemical Centre, Amersham, England and Sephadex G-25 (particle size 20–80 μ) was purchased from Pharmacia Fine Chemicals, Upsala, Sweden. Orcein was purchased from E. Merck, Germany. ATP and cytochalasin B were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

Isolation of actin like protein

The method used is that of Ghosh *et al.* (1988).

ELISA

Mouse monoclonal anti-actin antibody (Amersham, UK) was used in ELISA and a standard curve was constructed with rabbit muscle actin (Sigma) following the protocol supplied by Amersham. Microtiter plates were coated with different concentrations of purified mung bean seedling actin in 100 μl volume overnight at 4°C in Tris–HCl (pH 9·5). Nonspecific absorption was blocked with 1% bovine serum albumin in phosphate buffer saline (PBS) of the following standard composition, 137 mM NaCl, 2·7 mM KCl, 1·5 mM KH_2PO_4, 8 mM Na_2HPO_4. Anti-actin antibody diluted to 1:1000 in PBS was allowed to bind with the coated antigen at 37°C for 1 h. After thorough washing of the microtiter plates with PBS, protein A—enzyme conjugate at 1:10,000 dilution was allowed to hybridize at 37°C for 1 h. After washing ABTS was applied in each well (1 mM, 100 μl) as substrate. The colour development was monitored at 410 nm. All washings were done with PBS Tween-20.

Microscopic observation of cell division in mung bean seedling in the presence and absence of cytochalasin B

Mung bean seeds were allowed to germinate at 25°C for 24 h in a petridish on water saturated filter paper after surface sterilization with 1% sodium hypochlorite solution for 10–15 min followed by washing with distilled water twice or thrice and swelling. The germination was performed in dark. Immediately after germination, the root tips of the seedlings were immersed separately in 5 ml of cytochalasin B solution (2 and 50 μM respectively) for next 24 and 48 h, for the entry of the chemical inside the root-tip meristem. Three replicate sets were maintained for each treatment. The total length of each seedling was measured before and after cytochalasin B treatment and the mean values were recorded. The root tips were then excised, washed with water and fixed in acetic acid and ethanol (1:2) for 3 h and finally stained with 2% orcein:HCl (9:1) for 45 min. The root tips were finally squashed on a slide and observed under transmitted light microscope.

Binding of cytochalasin B with mung bean seedling actin

Binding of cytochalasin B with mung bean actin was done according to the method of Hummel and Dreyer (1962). Protein concentration of each fraction was measured according to the method of Lowry *et al.* (1951). Radioactivity was determined by spotting 200 μl of each fraction on glass fibre filters (Whatman GF/C) and counting the filters in a Beckman LS-1800 liquid scintillation counter.

Results

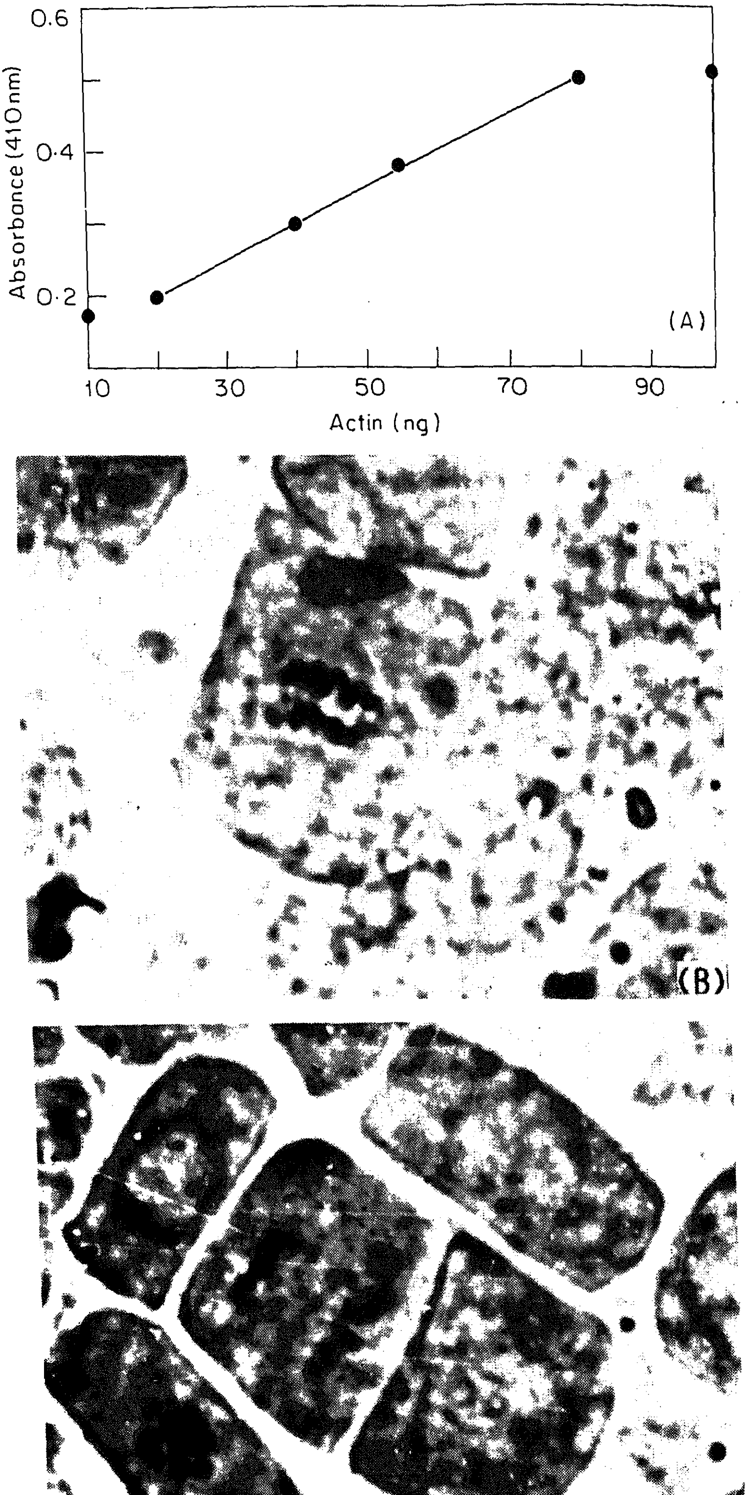
An actin like protein purified by Sepharose 4B column chromatography from mung bean seedling (Ghosh *et al.*, 1988) was analysed by the competitive ELISA and the results are depicted in table 1. A standard curve was constructed using pure actin from chicken gizzard (Sigma) (figure 1). Purified tubulin from goat brain was used as a nonspecific antigen. No activity could be measured when the same experiment was repeated with an antibody raised against the enzyme Inositol-l-phosphate dehydrogenase (obtained as gift from Mrs. M. Rudra, Biochemistry Department, Bose Institute).

The lethal dose of cytochalasin B was determined prior to studying its effect on cell division during germination of mung bean seeds. Pilot experiment using various amounts of cytochalasin B demonstrated that germination was completely inhibited

Table 1. ELISA for mung bean actin.

Origins	Concentration (in ng) added	Absorbance (410 nm)	Reactivity (%)
Chicken gizzard	20	0·301	100
	40	0·593	
Rabbit muscle	20	0·259	86
	40	0·502	86
Mung bean seedling	40	0·213	35
	80	0·369	31
Tubulin protein (+) from goat brain	40	0·031	5·2
	80	0·052	4·3

Assay was done as indicated in methods.
The values are average of 5 determinations.


0.6
0·4
0·2
Absorbance (410nm)
(A)
10
30
50
70
90
Actin (ng)
(B)

at a lethal dose of 500 μM cytochalasin B. Insignificant germination was observed at sublethal doses, which were close to the lethal dose. Doses like 2 and 50 μM were chosen for this study; germination was poor at 50 μM concentration when compared to that of control.

The normal cell division profile of the root tip cell of mung bean seedling is presented in figure 1B, C. Figure 1B shows the picture of an early anaphase, where chromosomes are separated from chromatids and movement of the chromosomes towards the pole has just started. Figure 1C represents the position of the chromosome in late anaphase where the chromosomes move closer to the poles.

When the mung bean seedlings were treated with cytochalasin B, a significant variation in cell division profile was observed under the microscope (figure 2A, B). The haphazard distribution of chromatids at the equitorial plane is observed in figure 2A. Actually in the presence of 50 μM cytochalasin B, the divisional frequency was less than the control set. Most of the (90%) dividing cells were in metaphase and less than 1% cells were in normal anaphase. Only 5% of the dividing cells showed chromatid separation, but no movement towards the pole was observed and chromatids were distributed randomly at the equitorial plane. After prolonged treatment (72 h) of cytochalasin B, the spindle attachment was totally lost and the chromosomes were displaced from their original equitorial plane (figure 2B). The effect of the drug cytochalasin B at 50 μm concentration on spindle attachment was found to be reversible as studied up to 24 h.

In the presence of 2 μM cytochalasin B, 75% of the mung bean seedling cells showed normal division and irregular spindle formation was observed in only 7% of the dividing cells. However less than 5% of the dividing cells showed chromatid separation, but without any movement. The length of the seedling before and after cytochalasin B treatment is presented in table 2.

The binding of actin (authentic) with cytochalasin B is a reversible process and hence all the binding parameters are measured on basis of equilibrium dialysis (Lin and Lin, 1978) as the routinely used filter disc assay method is unsuitable for studying the reversible binding. However, the binding nature of plant actin with cytochalasin B is still unknown. To study the binding of plant actin with cytochalasin B, the method of Hummel and Dreyer (1962) is chosen because the method is (i) rapid, (ii) maintains the protein ligand complex in equilibrium conditions and (iii) specially suitable for the detection of relatively weak binding. In this method, mung bean seedling F-actin fraction was made 10 μM with [^{3}H]cytochalasin B and applied to a gel filtration column (Sephadex G-25), equilibrated with the same concentration (10 μM) of [^{3}H]cytochalasin B and the equilibrium value *i.e.*, the base line level as evident from figure 3 are 10,000 cpm. The figure shows the elution profile of apparent cytochalasin binding, when actin was passed through the above mentioned column. As actin [^{3}H]cytochalasin B complex peak emerges at the excluded volume of the column, the total radioactivity in eluate rises above the equilibrium level at the position of actin elution. After the

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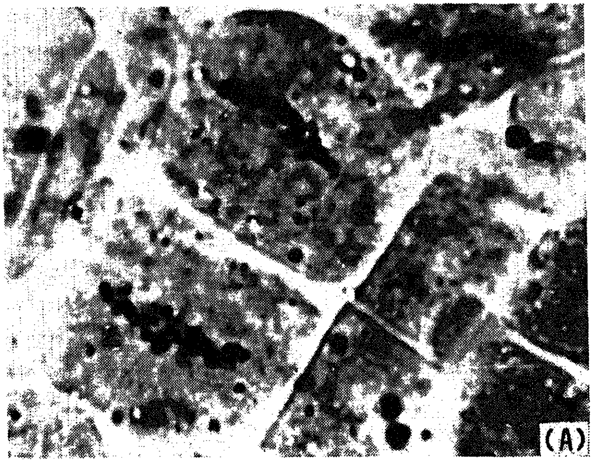
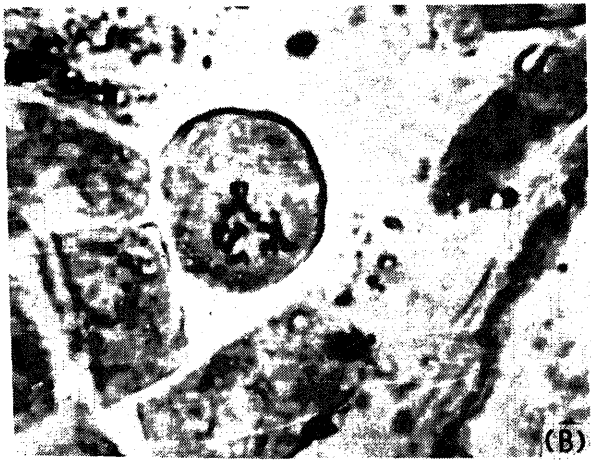
Figure 1. (A) Standard curve for ELISA of the chicken gizzard actin (Sigma) used for coating the microtiter plates. Antibody (Amersham) was raised against chicken gizzard actin. **(B)** Control root tip cells showing early anaphase stage. In each case 3 different sets of experiments were done and 500 cells were counted. **(C)** Control root tip cells showing the position of chromosome in late anaphase stage.


(A)


(B)

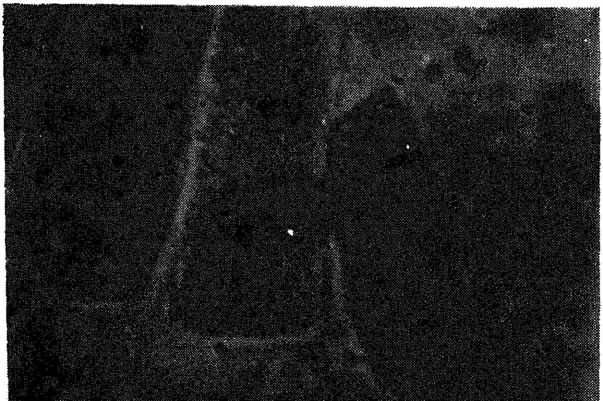


Table 2. Effect of cytochalasin B on the growth of the mung bean seedling.

	Length (mm)		
Time (h)	Control	+CB (2 μM) after 24 h	+CB (50 μM) after 24 h
24	2·5 ± 0·41	3·1 ± 0·15	2·76 ± 0·22
48	8·13 ± 0·22	7·96 ± 0·21	4·66 ± 0·13
72	17·3 ± 0·31	17·3 ± 0·23	8·1 ± 0·12

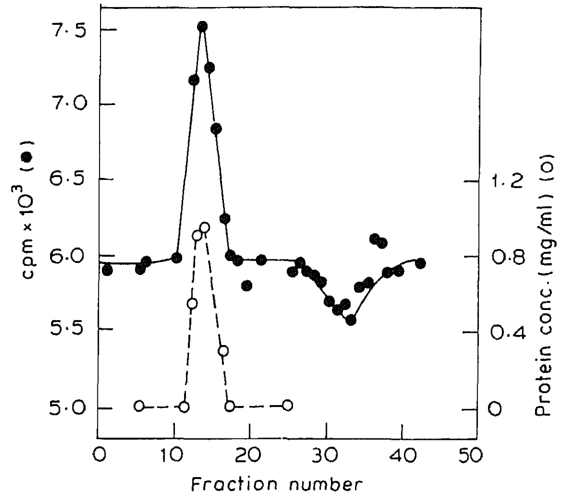


Figure 3. A Hummel-Dreyer column, Sephadex G-25 (25 × 1 cm) was used to measure the binding of cytochalasin B with mung bean seedling actin. The column was equilibrated with 10 mM imidazole-chloride pH 7·5 containing 4 mM $MgCl_2$ and 10 μM [^{3}H]-cytochalasin B. 0·6 ml of mung bean seedling F-actin (5·71 mg/ml) mixed with column buffer containing 20 μM [^{3}H]cytochalasin B in a 1 : 1 ratio was applied to this column, followed by the elution with the column buffer. Protein concentration of the fractions (0·6 ml) was estimated by Lowry's method (Lowry *et al.*, 1951). The radioactivity was determined by spotting 200 μl of each fraction on glass fibre filters (Whatman GF/C) and counting them in a Beckman LS-1800 liquid scintillation counter. All chromatography was performed at room temperature. Three different sets of experiments were done simultaneously. Mean value was plotted.

ak [^{3}H]cytochalasin B in the eluate decreases below the base line level
trough (which is equivalent to [^{3}H]cytochalasin B removed by protein
e amount of free [^{3}H]cytochalasin B removed from the column as
by this trough is equal to the amount of the drug found in the protein
dissociation constant (Kd) of the actin cytochalasin B complex was
from the following equation.

PAGE) along with purified rabbit actin in a parallel lane and finally stained with Commassie brilliant blue (R 250) (figure 4). After densitometric scanning, the peak area under actin region was measured and the actin concentration was calculated (table 3). The Kd value obtained in this case was $1{\cdot}2 \times 10^{-5}$ M (table 4).

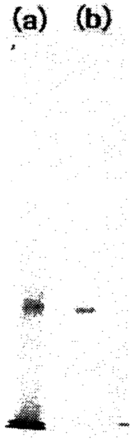


Figure 4. 7·5% SDS-PAG electrophoretic pattern of purified actin. (a) Mung bean actin 67 μg. (b) Rabbit actin (Sigma) 10 μg.

Table 3. Calculation of actin concentration from peak protein fraction by densitometric scanning.

Protein	Area under actin peak (sq. cm.)	Actin (μg/100 μl)	Actin concentration (μM) (M)
Purified rabbit muscle actin	3·15	20	4·7
Purified mung bean actin	1·98	12·6	3·0

Molecular weight of actin was considered as 42,000 Kd.
Densitometric scanning was performed using LKB 2202 ultrascan laser densitometer.

Table 4. Binding of cytochalasin B to actin like protein of mung bean seedling as measured by the Hummel-Dreyer method.

Column cytochalasin B (C)	Count at equilibrium line (cpm/200 μl)	Count above equilibrium line (cpm/200 μl)	Protein bound cytochalasin B (μM) (AC)	Peak actin (μM) (A)	Kd
10	6×10^3	$7{\cdot}5 \times 10^3$	2·5	3·09	$1{\cdot}2 \times 10^{-5}$

Discussion

The results show evidences for the presence of actin like protein in mung bean seedling. Two independent means of studies based on immunological method and ical cytochalasin B binding lead to the conclusion that this protein from higher nt has some similarity to actins from animal sources. However, studies on munochemical characterization of actin from higher plants where also it has not en possible to raise antibody against plant actin have been reported (Metcalf III *al.*, 1980; Ilker *et al.*, 1979). ELISA test with mung bean actin like protein shows t though some common antigenic sites exist between plant and animal actin, all sites are not identical or available for the interactions.

This actin like protein from mung bean seedling has a detectable cytochalasin B ding capacity with a Kd value $1{\cdot}2 \times 10^{-5}$ M. The binding of muscle actin with ochalasin B was measured using equilibrium dialysis and the Kd value was $^{-8}$ M, signifying much higher affinity. However, uptil now there is no other ort about the binding of plant actin with cytochalasin B to compare the binding racteristics of mung bean seedling actin with cytochalasin B. The inhibitory ct of cytochalasin B on *in vitro* actin polymerization from mung bean seedling s already shown (Ghosh *et al.*, 1988). Furthermore, the *in vivo* effect demonstrates t cytochalasin B at a sublethal dose (50 μM) blocks the chromosomal movement metaphase stage and growth of the seedling is also affected. It has been reported t cytochalasin B induces cessation of cytoplasmic streaming mediated by crofilament (Williamson, 1972; Bradley, 1973) and prevents normal telophase rientation movement which leads to abnormal division planes in dividing guard ther cell in Allium (Palevitz, 1980). The presence of actin containing crofilaments in mitotic spindle in plants have been reported conclusively on the sis of binding specifically to fluorescently labelled phallotoxins (Wulf *et al.*, 1979, sacreta *et al.*, 1982; Clayton and Lloyd, 1985), but this does not prove beyond ubt its involvement during the process. The mild and delayed inhibition of onion t mitosis by cytochalasin B even at 30 μg/ml (Thomas *et al.*, 1973) is quite tinct from the rapid growth inhibition accompanied by tip swelling and yploidy, induced by colchicine (Levan, 1938). Several studies (Hepler and evitz, 1974; Fonte *et al.*, 1978; Griffith and Pollard, 1982; Traas *et al.*, 1985) wed the possible involvement of actin in microtubule orientation most notably ring differentiation of trachery elements in *Zinnia* (Falconer and Seagull, 1985) l in the cytokinetic apparatus in general (Clayton and Lloyd, 1985; Gunning and ck, 1985; Palevitz, 1987). In fact, the effect of cytochalasin B in arresting mitosis the present case might be due to indirect effects such as involvement of actin in crotubule orientation or the many known effects of cytochalasin on plant cell ctions, which might well contribute to an arrest in mitosis.

Correlating all the above facts, it is indicative that for normal cell division, there st be some involvement of actin association with chromosomal movement. In s case if the drug cytochalasin B enters inside the cell, it is expected to bind the in molecules (confirmed from the binding study), eventually block the ymerization of actin protomer to the fibrous form (judged from the *in vitro* ymerization assay) and finally stop the chromosomal movement at metaphase ge (as observed in figure 2A). It is also observed that cytochalasin B treated ng bean seedling cell extract is less potent in polymerizing ability, when npared with untreated cell extract (Ghosh *et al.*, 1988). It is perhaps due to the ping of the actin molecule by cytochalasin B.

n dividing rat Kangaroo cell, actin was found to be present in discrete bundles necting individual chromosomes to the poles (Sanger, 1975). Earlier, the movement of chromosome during cell division (Inoue and Sato, 1967). Since both actin and microtubules are connected to the chromosome, there may be some co-operation between the two proteins to produce the necessary contractile force for chromosome movement (Brown and Spudich, 1979). The idea that actin containing filament might interact with microtubules to disrupt the mitotic spindle has been considered by Forer (1974), Nicklas (1977) and Oakley and Heath (1978). Forer *et al.* (1979) have shown in *Haemanthus* endosperm that microtubules can interact with actin containing filaments which produce force during mitosis. It is relevant to point out that our observations are consistent with those of others (Forer *et al.*, 1979; Forer and Jackson, 1979) in showing that actin is playing an active role in chromosome movement along with tubulin.

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Comparison of biotin binding protein of pregnant rat serum with rat serum albumin

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Abstract. The purified biotin binding protein of pregnant rat serum was shown to be immunologically similar to rat serum albumin as assessed by a sensitive radioimmunoassay. In radioimmunoassay for rat biotin binding protein, the binding of [^{125}I] rat biotin binding protein to anti-chicken egg yolk biotin binding protein antibodies was displaced by both rat serum (10–100 nl) and purified rat serum albumin (0·1–10 ng). Similarly, in radioimmunoassay for rat serum albumin the binding of [^{125}I] rat serum albumin to either anti-rat serum albumin antibodies or anti-chicken egg yolk biotin binding protein antibodies was displaced by unlabelled rat biotin binding protein at comparable concentration range (0·5–10 ng). Significant fractions of radioiodinated rat biotin binding protein and rat serum albumin bound to antibodies to chicken egg yolk biotin binding protein. In immature rats, the circulating half-lives of rat biotin binding protein and rat serum albumin were determined to be 12 and 17 h respectively. The rat biotin binding protein and rat serum albumin were analysed by techniques that exploit their physicochemical properties. They displayed similar electrophoretic mobilities in alkaline as well as denaturing sodium dodecyl sulphate-polyacrylamide gels. However, in non-equilibrium pH gradient polyacrylamide gel electrophoresis, they resolved clearly. In two-dimensional tryptic peptide map analysis, the two proteins showed similarities as well as significant differences in the relative distribution patterns of their iodopeptides. These results showed that the primary structure of rat biotin binding protein and rat serum albumin were different in finer details despite the fact that they shared significant immunological cross-reactivity.

Keywords. Biotin binding protein (chicken, rat); rat serum albumin; immunological-crossreactivity.

Introduction

In some mammalian species, carrier proteins for water-soluble vitamins present in maternal circulation during gestation are obligatorily required for the transplacental transport of vitamins to the developing fetus (Adiga and Murty, 1983; Adiga *et al.*, 1986). In the rat, carrier proteins for riboflavin (Murty and Adiga, 1982a) and thiamin (Malathy and Adiga, 1985) are estrogen-inducible proteins of hepatic origin and immunological intereference with the functioning of these vitamin carriers during pregnancy is detrimental to embryo development, culminating in pregnancy termination (Muniyappa and Adiga, 1980; Murty and Adiga, 1981, 1982b; Malathy 1985). Such selective immunoneutralisation of vitamin carriers bring about drastic curtailment of transport of the particular vitamin to the developing embryo and hence early embryonic mortality (Murty and Adiga, 1981; Surolia *et al.*, 1985).

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Abbreviations used: BBP, Biotin binding protein; rBBP, rat biotin-binding protein; RIA, radioimmunoassay; RSA, rat serum albumin; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; SA, serum albumin; M_r, molecular weight.

Recently, biotin binding protein (BBP) was purified from pregnant rat serum (Seshagiri and Adiga, 1987) and it's functional significance during pregnancy was demonstrated (Seshagiri and Adiga, 1989). This rat BBP (rBBP) is a glycoprotein of molecular weight (M_r) 66,000, has pI of 4·1 and specifically binds [^{14}C] biotin. This protein exhibited immunological cross-reactivity with the well characterised chicken egg yolk BBP (yolk BBP). The rBBP was isolated in significant amounts from the sera of pregnant or estrogen-treated females but not from those of the males, implying thereby the involvement of the steroid hormone in the elaboration of this protein (Seshagiri and Adiga, 1987). Therefore, it was considered desirable to develop a specific radioimmunoassay (RIA) for rBBP for quantification to explore the specificity and the pattern of hormonal modulation of rBBP induction, on lines similar to induction of avian egg yolk BBP (Murty and Adiga, 1985). During such attempts, it was surprising to find that rBBP and rat serum albumin (RSA) exhibited significant immunological cross-reactivity in RIA. Subsequent experiments however revealed that rBBP can be distinguished from RSA in terms of certain physicochemical properties.

Materials and methods

Albino rats (Wistar) of the Institute Colony were fed a pelleted diet (Hindustan Lever, Bombay) and water *ad libitum* and exposed to a 14:10 h light-dark schedule. Purification of BBP from the sera of pregnant rats was accomplished according to the published procedure (Seshagiri and Adiga, 1987). Antisera against yolk BBP and RSA (raised in rabbits) were obtained by standard procedures described earlier (Murty and Adiga, 1984). Carrier-free Na [^{125}I] were obtained from Amersham, UK. Iodogen (1,3,4,6-tetrachloro-3α, 6α-diphenyl glycouril) was obtained from Pierce Chemicals, Rockford, USA. The sources of other chemicals and reagents were the same as described earlier (Seshagiri and Adiga, 1987).

Heterologous RIA for rBBP

In view of the poor yield coupled with the batch to batch variation in purity and biological activity, monospecific antiserum to rBBP could not be obtained and therefore, heterologous RIA was developed for rBBP quantification employing rabbit anti-yolk BBP antibodies. Iodination of rBBP was performed at room temperature in the presence of iodogen (Fraker and Speck, 1978). The iodinated protein was resolved from free iodine by gel-filtration chromatography on a Sephadex G-75 column pre-equilibrated with phosphate buffered saline containing 0·3% gelatin. The immunoassay procedure employed is as described earlier (Malathy and Adiga, 1985) and used an antiserum dilution capable of binding 25–40% of the radio-labelled protein and the double antibody precipitation to separate bound from free antigen.

Determination of half-life of rat BBP

ure male rats *via* intra-cardiac route. Blood samples withdrawn at suitable
ls were counted for radioactivity. A least square fit of the data was
ted.

ctrophoresis

phoresis was carried out on 7·5% polyacrylamide gels (PAGE) at pH 8·3,
, 1964). Sodium dodecyl sulphate (SDS)-PAGE was carried out on 7·5% gels
ing to the standard procedure (Weber and Osborn, 1969). The proteins on
ls were stained with Coomassie brilliant blue R-250 and destained with
nol/glacial acetic acid/water (40:10:50, v/v). Non-equilibrium pH gradient
mensional PAGE was performed as follows (O'Farrell *et al.*, 1977). Briefly,
BBP and/or [^{125}I] RSA was electrophoresed on isoelectric focussing gels for
in capillary tubes (200 μl) followed by SDS-PAGE (7·5%). After this, the gel
eated with 12% (w/v) trichloroacetic acid for 1 h and dried using a gel-drier
nacia, Sweden, Model GSD-4) prior to exposing the gel to an X-ray film for
diography.

imensional tryptic peptide mapping of [^{125}*I*]*-labelled proteins*

nalysis was performed according to the published procedure (Elder *et al.*,
Briefly, the stained protein bands resolved by SDS-PAGE were excised and
otein trapped in the gel was subjected to *in situ* radioiodination by
mine T method (Greenwood *et al.*, 1963). The radioiodinated protein was
ubjected to protease digestion for 12 h at 37°C with TPCK-trypsin (50 μg in
of 0·1 M ammonium bicarbonate). The released peptides were quantitatively
red, concentrated and then subjected to electrophoresis (100 V for 90 min) on
se-coated plastic sheets using the buffer, glacial acetic acid/formic acid/water
0:800, v/v). This was followed by chromatography in the second dimension
the solvent system butanol/pyridine/acetic acid/water (32·5:25:5:2, v/v). The
were air-dried and then exposed to X-ray films for autoradiography.

s

rologous RIA to rBBP was developed employing anti-yolk BBP antibodies.
tandard curve of rBBP-RIA had a linearity range of 500 pg–10 ng with a
vity of 0·5 ng per tube (figure 1A). Both male and estrogen-treated female rat
could displace the binding of [^{125}I]rBBP from the heterologous anti-yolk
ntibodies in a parallel fashion; nearly 80% inhibition of binding could be
ed by 100 nl of either of the sera tested (figure 1A). The cross-reacting protein
sera, present in such an high concentration, was suspected to be RSA, since
$_r$ of the isolated rBBP was close to that of RSA (66,000). This premise was
r strengthened by the observation (figure 1B) regarding the dose-dependent
cement of [^{125}I]rBBP from anti-yolk BBP antibodies by RSA (either
ercial preparation or that purified by passing through an immuno-affinity
n of anti-yolk BBP-IgG coupled to Sepharose 4B).
rder to determine whether rBBP also inhibits the binding of RSA to anti-

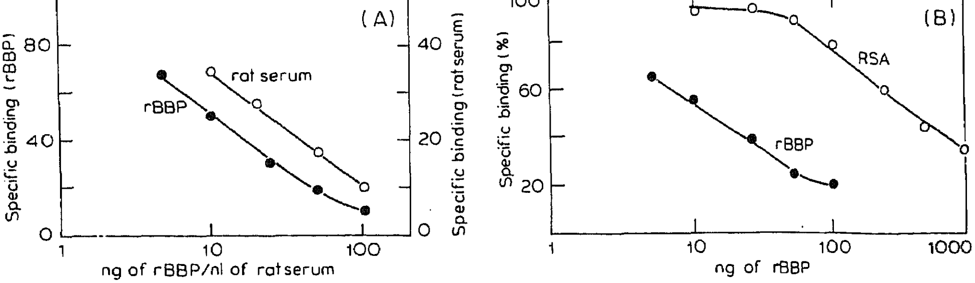

Figure 1. Displacement of [^{125}I] rBBP binding to yolk BBP antibodies by rat serum (A) and rat serum albumin (B). The dilution of the yolk BBP antiserum used was 1:1000.

RSA antibodies, an RIA for RSA was developed (figure 2A). This had a linearity range of 250 pg–10 ng and a sensitivity of 0·25 ng per tube. By utilising this assay, it could be shown that purified rBBP could significantly displace the binding of [^{125}I] RSA from anti-RSA antibodies (figure 2A) and the concentration of rBBP required for a 50% displacement was approximately 3-fold higher than that required by RSA (figure 2A), thereby implying that both rBBP and RSA appear to recognise similar population of antibodies. In order to further substantiate this observation, binding of iodinated rBBP and RSA to both the anti-RSA antibodies and the anti-yolk BBP antibodies was determined. From figure 3A, it was evident that at 1:100 dilution, the extents of binding of [^{125}I]rBBP to anti-yolk BBP antibodies and to anti-RSA antibodies were 35 and 25% respectively. On the other hand, at 1:100 dilution, the extents of binding of [^{125}I]RSA to anti-RSA antibodies and to anti-yolk BBP antibodies were 90 and 80% respectively (figure 3B). This consistent finding of [^{125}I]RSA binding to anti-yolk BBP antibodies enabled construction of a standard curve for RSA using anti-yolk BBP antibodies. The linearity range of the standard curve thus obtained was 200 pg–3 ng with a sensitivity of 0·2 ng per tube (figure 2B). In this experiment also, [^{125}I]RSA binding to anti-yolk BBP antibodies could be

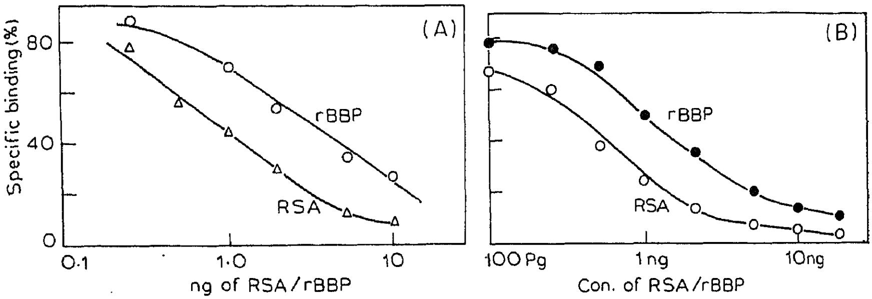

Figure 2. Displacement of [^{125}I] RSA binding to either RSA antibodies (A) or yolk BBP antibodies (B) by rBBP. The dilutions of RSA antiserum and yolk BBP antiserum used were 1:100,000 and 1:1000 respectively.

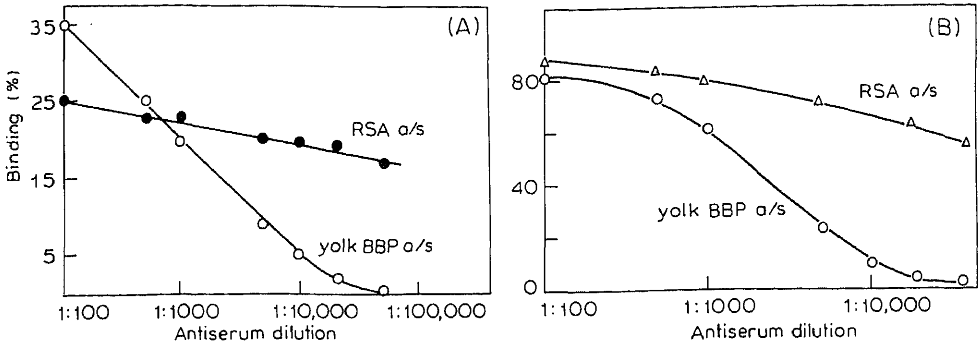

Figure 3. Binding of [^{125}I] rBBP (A) or [^{125}I] RSA (B) to either yolk antibodies or RSA antibodies. The input radioactivity for both the radioiodinated proteins was approx. 30,000 cpm.

displaced by unlabelled rBBP; an approximately 3-fold higher concentration of rBBP *vis-a-vis* RSA was required for 50% displacement (figure 2B).

Rate of disappearance of rBBP and RSA

The data of figure 4, show the kinetics of disappearance of the injected [^{125}I]rBBP and [^{125}I]RSA from the circulation of immature rats. The rates of disappearance

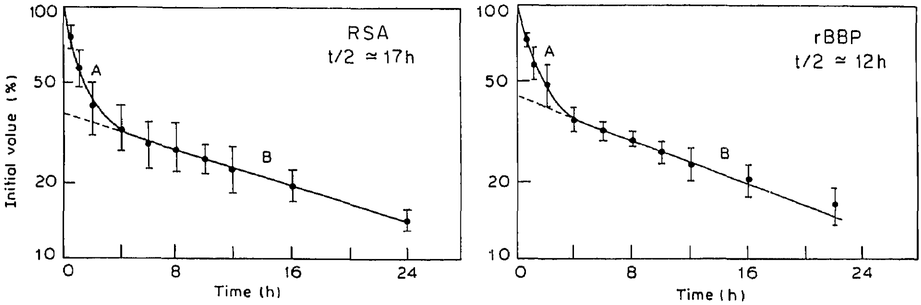

Figure 4. Rate of disappearance of [^{125}I] labelled RSA and rBBP from the plasma of immature rats. Freshly prepared radio-labelled RSA or rBBP (approx. 5 lakhs cpm) was injected into immature male rats and at the indicated time intervals blood samples were collected and plasma prepared were monitored for radioactivity. The values represent mean and the vertical bars represent SD (n = 5). (A) Phase of rapid disappearance; (B), phase of catabolism.

of both the proteins exhibited a biphasic pattern, the rapid initial phase (A) presumably representing the dilution of the label with the extravascular space, which was then followed by a slow phase (B) of disappearance. The latter phase is considered to correspond to true catabolism. The slower phase followed a first order kinetics and the least square fit of the data yielded a half life of 12 and 17 h for rBBP and RSA, respectively.

Comparison of electrophoretic mobilities of rBBP and RSA

Resolution by alkaline PAGE (pH 8·3) as well as SDS-PAGE revealed that rBBP and RSA with similar mobilities migrated in both the types of gels with no discernible separation when applied either singly or as a mixture of these two proteins (figure 5). On the other hand, when a mixture of radioiodinated rBBP and RSA was subjected to resolution by two-dimensional non-equilibrium pH gradient PAGE, the autoradiograms revealed two distinct well separated spots (figure 6), the more acidic one corresponding to rBBP. These results clearly reveal a significant pI difference between rBBP and RSA.

Figure 5. Comparison of electrophoretic mobilities of rBBP and RSA. Alkaline (A) and SDS-denatured (B)—PAGE on 7·5% slab gels were performed as detailed in the text. Lanes 1–3 contained 20 μg of rBBP (1), a mixture of 15 μg each of rBBP and RSA (2) and 20 μg of RSA (3).

Two-dimensional tryptic peptide map analysis of radioiodinated rBBP and RSA

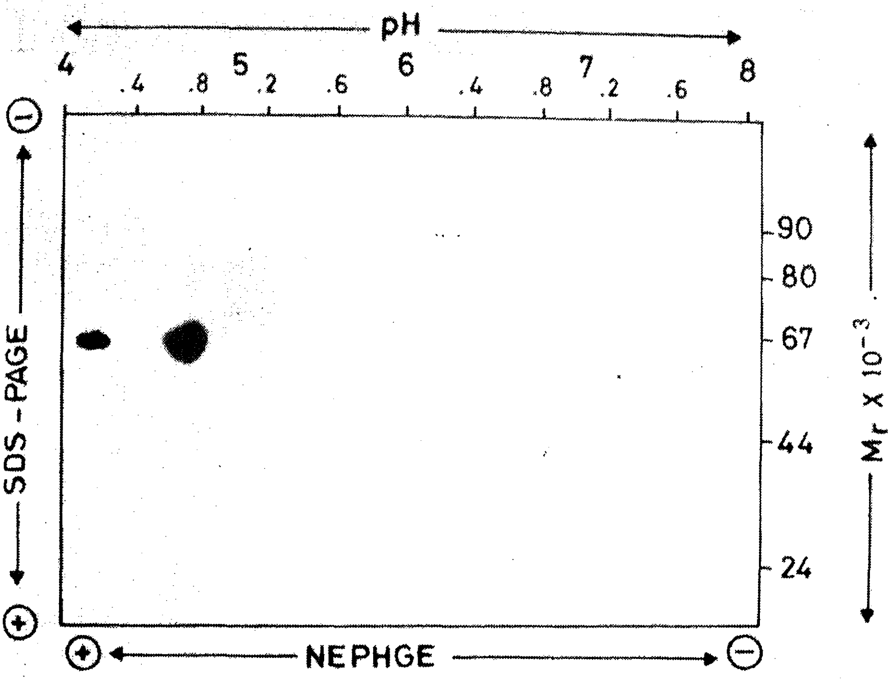

Figure 6. Two-dimensional non-equilibrium pH gradient PAGE of a mixture of radioiodinated rBBP and RSA. A mixture of radioiodinated rBBP and RSA (each approx. one million cpm) was subjected to isoelectric focusing (first dimension) followed by SDS-PAGE (second dimension) on 7·5% gels. An autoradiogram was prepared from this gel, as described in the text.

revealed that the overall pattern of distribution, especially the orientation of closely spaced peptides is not identical thereby indicating distinct differences in the primary structure between these two proteins. The only significant commonality between their peptide maps (figure 7) was with regard to the 3 peptides arranged in a triangle with comparable migrational properties; this might be indicative of similarities in limited amino acid sequences between rBBP and RSA.

Discussion

The primary aim contemplated in the present study was to develop a heterologous RIA for rBBP in order to examine the kinetics of hormonal induction of this protein. However, such an investigation was rendered difficult in view of the rather unexpected immunological cross-reactivity in RIA between rBBP and the abundant serum protein *viz.*, RSA. It was however noticed that other gross immunological methods such as immunodiffusion, immunoprecipitation and immunoelectrophoresis failed to show this cross-reactivity to any discernible extent (Seshagiri, P. B. and Adiga, P. R., unpublished results).

In displacement studies involving RIA of rBBP and RSA, the puzzling observation was the requirement of 3–5-fold lower concentration of unlabelled RSA

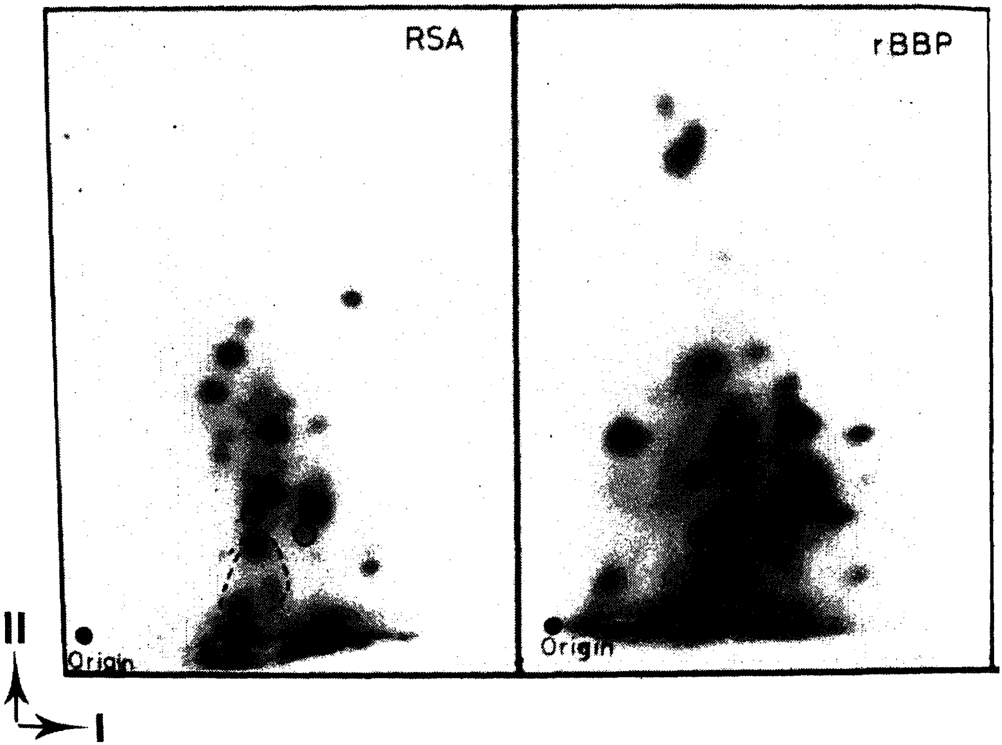

Figure 7. Two-dimensional tryptic peptide map analysis of radioiodinated RSA and rBBP. Radioiodinated tryptic peptides from RSA and rBBP were obtained as described in the text. These were dissolved in 10 μl of electrophoresis buffer, applied on one corner of the cellulose-coated plastic sheets and subjected to electrophoresis (first dimension: I), followed by chromatography (second dimension: II). The plates were air-dried and the autoradiograms were prepared. Peptides circled have comparable migrational properties in the maps of RSA and rBBP.

than that of rBBP for equivalent displacement (figures 1B and 2B). Although the precise reason for this finding is not clearly understood at present, it is conceivable that the extent of immunological inactivation of rBBP, absolute concentrations of the unlabelled proteins and their specific activities may be some of the major determining factors for such unusual behaviour. A similar situation was encountered in the case of displacement studies with different serum albumins (SAs). For example, complete inhibition of [^{125}I] rabbit SA binding to rabbit antibodies to human SA or bovine SA was obtained at 5-fold lesser concentration of unlabelled rabbit SA than the unlabelled human SA or bovine SA (Sakata and Atassi, 1981).

Another significant observation made in the present study was with regard to the recognition of antibodies specific to the protein of an avian origin (yolk BBP) by an g due either to close higher order structural similarities or to a limited amino sequence relatedness that might exist between proteins. It would therefore seem such unusual cross-reactivity is attributable to sequence/conformation-specific oodies which could bind to the related sequence/domain in the heterologous ein (Todd *et al.*, 1982). Among other examples belonging to this category are A and α-feto protein (Rouslahti and Terry, 1976), (ii) RSA and a M_r 64,000 ein induced under compensatory muscular hypertrophy (Yamada *et al.*, 1984), BSA and inhibin-like substance (Duraiswamy, S., unpublished results), (iv) rat s fluid protein and SA (Skinner *et al.*, 1987) and (v) different SAs of species that n close evolutionary proximity (Wilson *et al.*, 1977).

ne fact that there is significant cross-reactivity between rBBP and RSA as aled by RIA using polyclonal antibodies suggests that they share major genic determinants. This strong immunological relatedness between rBBP and raises the discrete possibility that rBBP may represent a very minute fraction pecially modified form of RSA. It is not uncommon that evolutionary nanisms can convert a single protein with only one defined function in one ies into a family of complex proteins with many different functions in another ies. This is exemplified by the report that a small sub-population of human IgG ifically binds riboflavin with high-affinity, presumably acting as a carrier ein for riboflavin (Chang *et al.*, 1981).

order to unravel other aspects of similarities and differences between the two eins, rBBP and RSA were subjected to some of the techniques that exploit rences in physicochemical properties of these proteins. Notable among the ome of these experiments are (i) the clear-cut pI difference *i.e.*, 4·1 for rBBP and or RSA (figure 6), also sustained by published reports (Seshagiri and Adiga, ; Calm, 1975) and (ii) significant changes in the overall distribution patterns of ic peptide maps of rBBP and RSA (figure 7). Other differences that differentiate e 1) between the two proteins are (i) detection of rBBP in circulation only r experimental estrogenisation (Seshagiri and Adiga, 1987) under such rimental conditions RSA in fact experience deinduction by estrogen manaban, G., unpublished results), (ii) glycosylated nature of rBBP unlike (Calm, 1975), (iii) different kinetics of disappearance of the injected [^{125}I] ? and [^{125}I]RSA (t/2: 12 and 17 h respectively, figure 4) and (iv) the recent ence from this laboratory (Seshagiri and Adiga, 1989) that functionally, rBBP is rent from RSA. The last mentioned finding stems from the observation that e immunoneutralisation of BBP in pregnant female rats resulted in early fetal rption without affecting the maternal health. This finding clearly discriminated from SA in terms of the functional role during pregnancy in the rat. The sum of these observations is that while rBBP and RSA have several immunological erties in common, they do exhibit many differences in physicochemical erties and more importantly in the functional property (table 1).

is tempting to speculate that the two proteins might have had a common n and their ancestral gene might have undergone modification by gene ication during evolution; during this process, the rBBP gene might have ired some additional special features like responsiveness to the steroid

Table 1. Comparison of rBBP and RSA.

Property	rBBP[a]	RSA[a]
Estrogen dependence	Induced	Deinduced
Binding to biotin AH-Sepharose?	Yes	No
Yield	Low[b]	—
Glycosylated?	Yes	No
pI	4·1	4·9
Specific [^{14}C]biotin binding	Yes	No
Tryptic peptide maps	Different	Different
Gross immunological cross-reactivity with anti-yolk BBP antiserum	Yes	No
Half-life (t/2) in circulation (h)	12	17
Role in embryonic development	Yes	No

[a] Information on rBBP and RSA, listed in these two columns, is obtained from this paper and from few other publications (Calm, 1975; Seshagiri and Adiga, 1989).

[b] The yield of rBBP is approximately 800 μg/100 ml serum (Seshagiri and Adiga, 1987).

biotin transport during pregnancy. It is pertinent to recall at this stage that vitamin D binding protein of human serum has been recognised as one of the members of the albumin gene family (Cook and Davis, 1985; Yang *et al.*, 1985).

Although the above hypothesis on the mode of evolution of rBBP gene remains to be purely speculative at this juncture, with the recruitment of more sophisticated tools of recombinant DNA technology, a better understanding of rBBP/RSA gene organisation and their mode of evolution from their ancestral gene may become a reality.

Acknowledgements

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duction of hepatic drug metabolizing enzymes by DL-methionine in ts

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Abstract. A single intraperitoneal injection of DL-methionine (500 mg/kg body wt.) to adult male Wistar rats was shown to significantly induce all the components of the hepatic microsomal mixed function oxidase system such as NADPH cytochrome *C* reductase activity, cytochromes P-450 and b_5, as well as activities of drug metabolizing enzymes such as aminopyrine demethylase and uridine 5′-diphosphate-glucuronosyltransferase. Combined administration of nicotinamide (250 mg/kg body wt.) and DL-methionine (500 mg/kg body wt.) was shown to bring about an additional increase (25–30%) in the activities of these enzymes as compared to their induction on independent administration of the two endobiotics. In rats bearing Yoshida sarcoma (ascites) tumour as well as in normal rats injected with serum from tumour bearing animals, the decreased activities of hepatic mixed function oxidases could be restored to their normal levels by administration of DL-methionine (500 mg/kg body wt.) to these rats. Whereas actinomycin D (1 mg/kg body wt.) had no effect on the increased incorporation of [^{14}C] labelled leucine into microsomal proteins following administration of nicotinamide, the enhanced incorporation of the label following DL-methionine administration was completely inhibited by the same dose of actinomycin D. Administration of cycloheximide (0·5 mg/kg body wt.) to rats could completely inhibit the increased incorporation of [^{14}C] leucine into hepatic microsomal proteins following independent administration of nicotinamide and DL-methionine. Similar inhibitory pattern with actinomycin D and cycloheximide was also demonstrated in case of induction of NADPH cytochrome *C* reductase activity by both these endobiotics.

Keywords. Mixed function oxidases; drug metabolizing enzymes; induction by methionine; tumour-bearing rats; host livers; methionine and nicotinamide; combined effect.

roduction

luction of drug metabolizing enzymes by exogenous compounds like drugs, cinogens and environmental chemicals is well documented using experimental mal systems (Parke, 1975; Depierre and Ernster, 1978; Conney, 1986; Okey *et al.*, 36). Several naturally occurring nutrients have also been shown to enhance the ivities of drug metabolizing enzymes (Mitoma *et al.*, 1969; Parke and Rahman, 59; Feuer, 1970; Wattenberg, 1972; Parke *et al.*, 1974; Hsiao *et al.*, 1975; Hvang *al.*, 1981). Earlier studies from this laboratory demonstrated that a single :aperitoneal (i.p.) administration of nicotinamide, a vitamin and an endobiotic ıld effectively bring about induction of all the components of hepatic mixed ction oxidases (MFO) system such as cytochromes P-450, b_5 and NADPH ochrome *C* reductase activity (Kamat *et al.*, 1980). The activities of hepatic :rosomal arylhydrocarbon hydroxylase, aminopyrine demethylase as well as drug ıjugating enzymes like uridine 5′-diphosphate (UDP)-glucuronosyltransferase

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and the soluble glutathione-S transferase (Kamat *et al.*, 1980; Mhatre *et al.*, 1983) were also shown to be effectively induced by nicotinamide in adult Wistar rats. This induction of hepatic drug metabolizing enzymes by nicotinamide could not be precisely explained, however since nicotinamide is readily metabolized to N_1-methylnicotinamide and since the methyl groups for the methylation originate from methionine through S-adenosylmethionine, effect of methionine administration to rats on possible induction of hepatic NADPH cytochrome *C* reductase activity was investigated. It was demonstrated that administration of DL-methionine (200 mg/kg body wt.) to rats could significantly induce the enzyme activity and further, when given in combination with nicotinamide, brought about a significant additional increase in the enzyme activity as compared to its induction on independent administration of nicotinamide and DL-methionine (Kamat *et al.*, 1980). Whatever the possible explanation for the induction phenomenon by these endobiotics, these observations prompted further studies on the induction of drug metabolizing enzymes by DL-methionine independently and in combination with nicotinamide in normal healthy rats. Further, induction of these enzyme activities was also applied to correct the decreased activities of drug metabolizing enzymes in host livers of rats bearing Yoshida sarcoma (ascites) tumour.

Materials and methods

Chemicals

Nicotinamide adenine dinucleotide phosphate-reduced form, (NADPH), cytochrome *C*, actinomycin D, cycloheximide, nicotinamide, and DL-methionine were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. 4-Dimethyl amino antipyrine (aminopyrine) was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, USA. DL-[1-^{14}C]-leucine (44·65 mCi/mmol) was obtained from Isotope Division, BARC, Trombay. All other chemicals used were obtained from BDH Chemicals Division, Glindia Ltd., Bombay.

Treatment of animals with DL-methionine and nicotinamide

Adult male Wistar rats (8–10 weeks old), unless specifically stated otherwise, were utilized in these studies. DL-Methionine (500 mg/kg body wt. i.p.) was administered to normal healthy rats (200–250 g) 24 h prior to killing. For dose-response pattern of enzyme induction, DL-methionine was administered to adult rats in two doses (250 mg and 500 mg/kg body wt.). Comparative effect of nicotinamide and DL-methionine was studied by injecting nicotinamide (250 mg/kg body wt.) and DL-methionine (500 mg/kg body wt.) to rats, independently and in combination. The animals were sacrificed 24 h after the injection. The combination treatment implies administration of nicotinamide and DL-methionine in quick succession.

Tumour transplantation

A group of Wistar rats (8–10 weeks old, weighing 250 g) was injected with Yoshida sarcoma ascites cells suspension (1 ml each) under asceptic conditions from the iously transplanted rats. This tumour grows very fast, killing the host animals n 6–7 days after transplantation. For studies on rats bearing Yoshida sarcoma es) tumour, DL-methionine (500 mg/kg body wt.) was administered on 3rd day tumour transplantation 24 h prior to killing.

tion of serum from tumour-bearing rats ·

m withdrawn from Yoshida sarcoma (ascites) tumour-bearing rats was injected each) to normal adult rats, 24 h prior to killing.

] *Leucine incorporation studies and effect of actinomycin-D and cycloheximide*

rporation of [^{14}C] labelled leucine into total hepatic microsomal proteins was ed 24 h after DL-methionine administration to male rats (500 mg/kg body wt. Actinomycin-D in two doses (1 and 0·1 mg/kg body wt. i.p.) and cycloheximide ng/kg body wt.) were separately injected to rats immediately after DL-ionine in independent experiments. DL-[1-^{14}C]-leucine (16 μCi/100 g body wt.) administered intraperitoneally to rats 1 h prior to killing. Hepatic microsomal ension (0·2 ml) was spotted on Whatman filter paper (3 mm thick) strips × 1·2 cm) which were further processed by the method of Mans and Novelli) as modified by Roodyn *et al.* (1965) prior to counting.

y procedures

rol and experimental tumour-bearing rats were killed by cervical dislocation. s were removed, chilled in ice and immediately washed with 0·154 M KCl wed by homogenization in the same medium. Hepatic microsomal preparations obtained according to the method of Berezney *et al.* (1972). Assay of NADPH hrome *C* reductase was carried out according to Williams and Kamin (1962). reduction of cytochrome *C* was calculated on the basis of molar absorbance given by Margoliash and Frohwirt (1959). The contents of cytochromes P-450 b_5 were determined as described by Omura and Sato (1964). Aminopyrine thylase activity was measured by the method of Muakkassa and Yang (1981). -glucuronosyl transferase activity was assayed according to the procedure of pacher *et al.* (1962) using *p*-nitrophenol as its substrate. Estimation of osomal proteins was carried out by the method of Lowry *et al.* (1951) using alline bovine serum albumin as the standard.

lts and discussion

dose and time response patterns of induction of hepatic MFO activity by DL-ionine are given in figures 1 and 2. It was observed (figure 1) that although the mum induction of NADPH cytochrome *C* reductase activity could be obtained the dose of 250 mg/kg body wt. a higher dose of 500 mg/kg body wt. was red to induce the levels of cytochromes P-450 and b_5. The reason for this ence in doses is not yet clear. It is evident from figure 2 that the maximum ction is at 24 h interval after the administration of DL-methionine. Figure 3

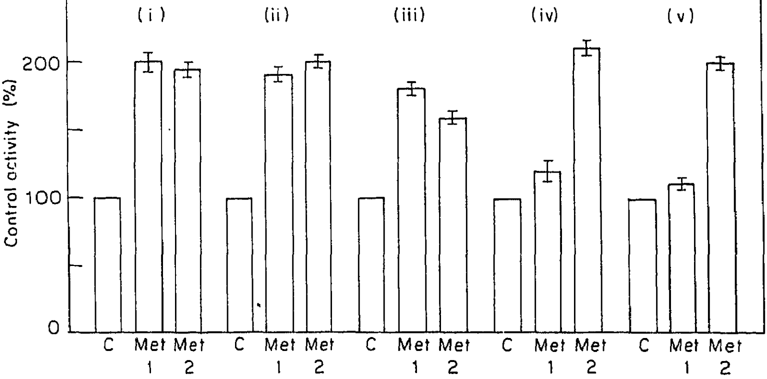

Figure 1. Effect of DL-methionine administration on the hepatic microsomal cytochromes P-450, b_5 and activities of other drug metabolizing enzymes in male rats. Results are expressed as per cent of control activity. Met 1 and Met 2, DL-methionine 250 and 500 mg/kg body wt. respectively. (i), NADPH cytochrome *C* reductase; (ii), aminopyrine demethylase; (iii), UDP-glucuronosyltransferase; (iv), cytochrome P-450; (v), cytochrome b_5. Each value represents mean ± SE of at least 5 independent experiments.

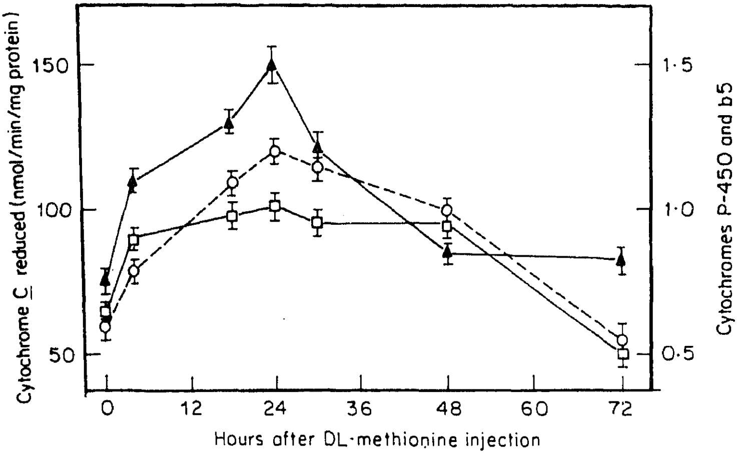

Figure 2. Hepatic microsomal NADPH cytochrome *C* reductase activity and the contents of cytochromes P-450 and b_5 in male rats treated with DL-methionine (500 mg/kg body wt. i.p.) at various time intervals. (▲), NADPH cytochrome *C* reductase; (○), Cytochrome P-450; (□), Cytochrome b_5. Each value represents mean ± SE of at least 5 independent experiments.

represents the effect of DL-methionine (500 mg/kg body wt.) administration to rats in the age group of 6, 7 and 8 weeks on MFO components. It is evident that the increase in the levels of cytochromes P-450 and b_5 as well as in NADPH cytochrome *C* reductase activity starts around 6 weeks of age and reaches maximum in 8 weeks old rats. Prior to 6 weeks no such induction of these enzymes could be observed (not shown in the figure). This difference may be attributable to the hormonal status of the animals. The induction pattern of hepatic microsomal MFO components as well as the activity of conjugating enzyme UDP-glucuronosyltransferase by independent and combined administration of DL-methionine and

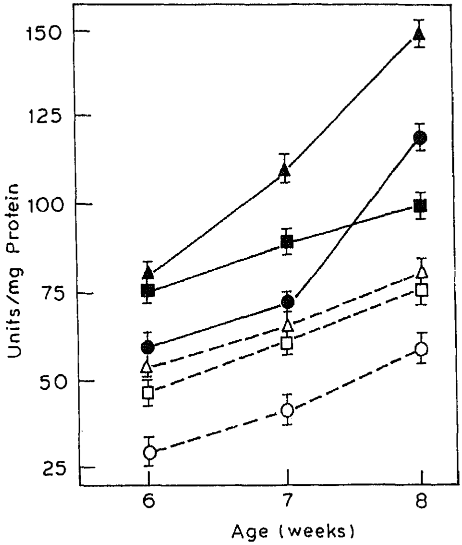

Figure 3. Effect of DL-methionine on hepatic microsomal NADPH cytochrome *C* reductase activity and cytochromes P-450 and b_5 in premature (6 weeks) and adult (8 weeks) rats. Control: (--△--), NADPH cytochrome *C* reductase; (--○--), cytochrome P-450; (--□--), cytochrome b_5. DL-methionine treated (500 mg/kg body wt. single i.p.); (▲), NADPH cytochrome *C* reductase; (●), cytochrome P-450; (■), cytochrome b_5. Each value represents mean ± SE of at least 5 independent experiments.

nicotinamide is given in figure 4. It is evident that combined administration of DL-methionine and nicotinamide brings about a significant increase in the activities of these enzymes over those observed after independent administration of both these endobiotics. This would suggest that the mechanisms of induction by DL-methionine and nicotinamide are different. Results of studies on [^{14}C] leucine incorporation into hepatic microsomal proteins and effects of actinomycin D and cycloheximide following independent administration of nicotinamide and DL-methionine to rats are summarized in table 1. It can be seen that in case of nicotinamide administration actinomycin D inhibited the incorporation of [^{14}C] leucine into microsomal proteins at the dose of 0·1 mg/kg body wt. which inhibits rRNA synthesis, but not at the higher dose of 1 mg/kg body wt. which inhibits transcription. DL-methionine on the other hand inhibits the incorporation of [^{14}C] leucine into microsomal proteins at both the doses of actinomycin D. As expected, cycloheximide blocks the increased incorporation of [^{14}C] leucine into microsomal proteins brought about independently by nicotinamide and DL-methionine. It can be seen that similar data was obtained with NADPH cytochrome *C* reductase activity with both these endobiotics after administration of actinomycin D. Although it would appear from the data in table 1 that DL-methionine induces the drug metabolizing enzymes at the transcriptional level while nicotinamide acts at the translational level, these results would have to be cautiously interpreted since more experimental evidence would be needed to draw such definite conclusion.

Induction of mixed function oxidases by both these endobiotics is interesting. Although nicotinamide is known to undergo methylation resulting in the formation of N_1-methyl nicotinamide, it has also been shown to undergo N-oxidation through

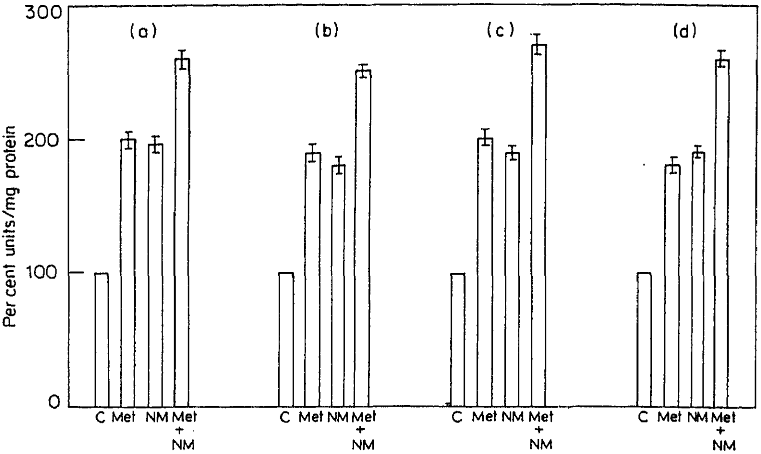

Figure 4. Effect of independent and combined administration of nicotinamide and DL-methionine on hepatic microsomal mixed function oxidase components and UDP-glucuronosyltransferase activity. The results are expressed as per cent of control activity. Nicotinamide (NM) (250 mg/kg body wt. single i.p.) and DL-methionine (Met) (500 mg/kg body wt. single i.p.) were administered singly and in combination to male rats in independent experiments. The activities of hepatic MFO components like NADPH cytochrome *C* reductase, cytochromes P-450, b_5 as well as the conjugating enzyme UDP-glucuronosyltransferase were determined 24 h after the treatment. (a), NADPH cytochrome *C* reductase; (b), cytochrome P-450; (c), cytochrome b_5; (d), UDP-glucuronosyltransferase. Each value represents mean ± SE of at least 5 independent experiments.

Table 1. Effect of actinomycin D and cycloheximide on incorporation of [^{14}C] leucine into hepatic microsomal proteins and the activity of hepatic NADPH cytochrome *C* reductase in DL-methionine and nicotinamide treated rats.

Treatment	Incorporation of [^{14}C]leucine into total microsomal proteins cpm/mg protein	NADPH-cytochrome *C* reductase activity nmol cytochrome *C* reduced/min/mg protein
Control	846 ± 21	50 ± 4
DL-methionine (500 mg/kg body wt.)	1485 ± 15	100 ± 7
DL-methionine + actinomycin D (1 mg/kg body wt.)	986 ± 10	45 ± 3
DL-methionine + actinomycin D (0·1 mg/kg body wt.)	1119 ± 25	36 ± 4
DL-methionine + cycloheximide (0·5 mg/kg body wt.)	1140 ± 21	30 ± 4
Nicotinamide (250 mg/kg body wt.)	1612 ± 50	100 ± 8
Nicotinamide + actinomycin D (1 mg/kg body wt.)	1732 ± 47	95 ± 7
Nicotinamide + actinomycin D (0·1mg/kg body wt.)	1185 ± 35	58 ± 2
Nicotinamide + cycloheximide (0·5 mg/kg body wt.)	1080 ± 30	42 ± 5

Experimental details are given in the text. Each value represents mean ± SE of atleast 4 independent experiments.

the MFO system (Nomura *et al.*, 1983). It may be that when nicotinamide is injected in high doses it brings about its own oxidation by induction of mixed function oxidases. DL-methionine is known to bring about methylation of γ-aminobutyric acid through S-adenosylmethionine to form γ-butyrobetaine which is further hydroxylated through the MFO system to form carnitine which participates in the mitochondrial fatty acid transport. It may be that formation of γ-butyrobetaine by methionine administration induces the MFO system. The induction of MFO by methionine is, however, in variance with the results of Magdalau *et al.* (1979) wherein it was demonstrated that continuous feeding of diet depleted in methionine and cysteine to rats kept on protein free diet brought about an increase in cytochrome P-450 as well as in UDP-glucuronosyltransferase activity. In this respect the effect of continuous feeding of DL-methionine to rats on the status of MFO needs to be examined in our experiments although the experimental conditions in our investigations are different from those of Magdalau *et al.* (1979).

Results of induction of hepatic drug metabolizing enzymes by DL-methionine in tumour-bearing rats are summarized in figures 5 and 6. It can be seen (figure 5) that contents of cytochromes P-450 and b_5 are significantly lower in host livers of rats bearing Yoshida sarcoma (ascites) tumour than those in controls. It is evident that when DL-methionine is administered to these rats, the contents of cytochromes P-450 and b_5 could be restored to their normal levels. The depletion in the levels of cytochromes P-450 and b_5 is also evident when serum from tumour-bearing rats is injected to normal healthy rats, and even this depletion is corrected by administration of DL-methionine to the serum injected rats. The inhibitory protein of MFO system from ascites cells has been isolated in our laboratory (unpublished

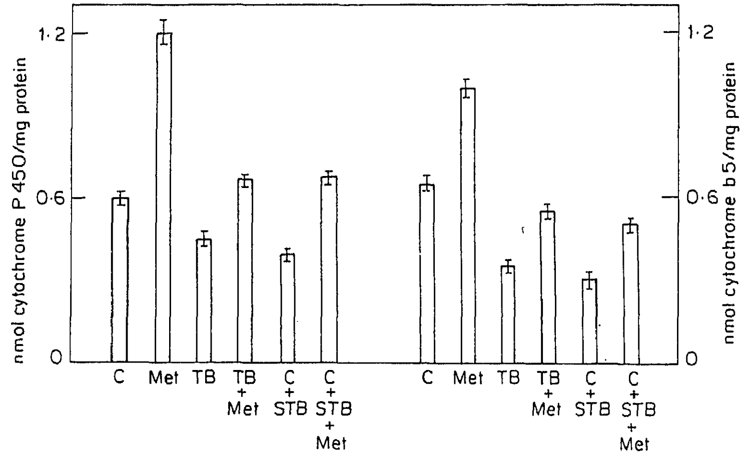

Figure 5. DL-methionine induced modification of decreased contents of hepatic microsomal cytochromes P-450 and b_5 in Yoshida sarcoma (ascites) tumour bearing rats and in normal rats injected with serum from tumour bearing rats. C, Control; Met, DL-methionine treated (500 mg/kg body wt. single i.p.); TB, tumour bearing (4 days transplant); TB + Met, tumour bearing (4 days transplant) treated with DL-methionine (500 mg/kg body wt. single i.p.); C + STB, normal healthy rats treated with serum from tumour bearing rats. C + STB + Met, DL-methionine (500 mg/kg body wt. single i.p.) was simultaneously injected to normal healthy rats injected with serum from tumour bearing rats. Each value represents mean ± SE of at least 5 independent experiments.

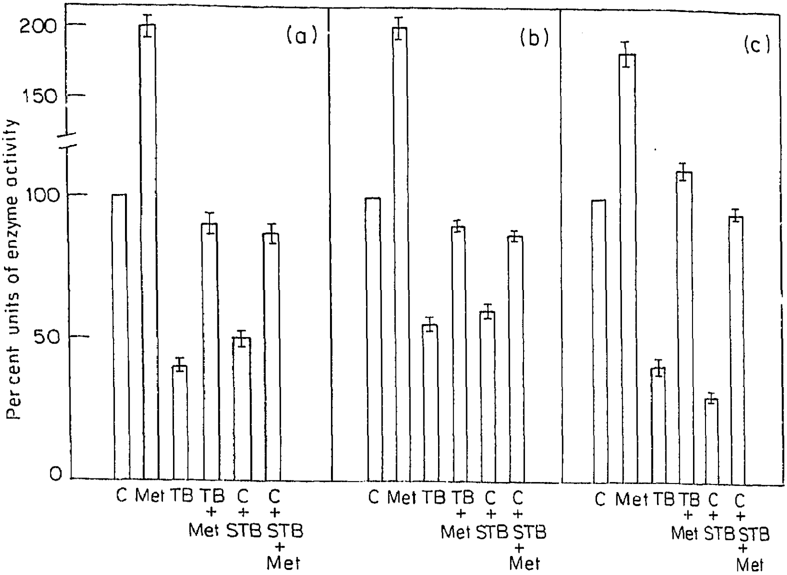

Figure 6. DL-methionine induced modification of decreased activities of hepatic microsomal drug metabolizing enzymes in Yoshida sarcoma (ascites) tumour bearing rats and in normal rats injected with serum from tumour bearing rats. The results are expressed as per cent of control activity. C, Control; Met, DL-methionine (500 mg/kg body wt. single i.p.) treated; TB, tumour bearing (4 days transplant); TB+Met, tumour bearing (4 days transplant) treated with DL-methionine (500 mg/kg body wt. single i.p.); C+STB, normal healthy rats treated with serum from tumour bearing rats. C+STB+Met, DL-methionine (500 mg/kg body wt. single i.p.) was simultaneously injected to normal healthy rats injected with serum from tumours bearing rats. (a), NADPH cytochrome C reductase; (b), Aminopyrine demethylase; (c), UDP-glucuronosyltransferase. Each value represents mean ± SE of at least 5 independent experiments.

results) and these experiments showed that the induction of MFO by DL-methionine is not affected by the presence of the inhibitory protein. Similar results with the administration of DL-methionine to tumour bearing rats as well as to control rats administered with serum from tumour bearing animals were obtained in respect of NADPH cytochrome *C* reductase, aminopyrine demethylase and UDP-glucuronosyltransferase activities (figure 6). These results are similar to our observations on application of nicotinamide-induced MFO system in tumour bearing rats (Kamat *et al.*, 1987). Nicotinamide and DL-methionine induced increased activities of drug metabolising enzymes are thus, novel systems suggesting their possible use in reducing toxicity during cancer chemotherapy.

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Effect of α-tocopherol on doxorubicin induced alterations in glucose metabolism—A pilot study

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Abstract. The effect of doxorubicin on glucose metabolism was studied in rats with or without the supplementation of α-tocopherol. Rats were treated with doxorubicin, 2 mg/kg body wt. (intravenously), twice a week, for 6 weeks. α-Tocopherol (400 mg/kg body wt.) was co-administered orally for 2 months. Glycolysis was found to be increased with a significant decrease in the activities of tricarboxylic acid cycle enzymes. A significant increase in liver glycogen was noted in doxorubicin treated rats. Activities of glycogen phosphorylase, glucose-6-phosphate dehydrogenase and glucose-6-phosphatase were found to be decreased.

α-Tocopherol co-administration was found to reduce the alterations in the above mentioned enzyme activities. The results are discussed with reference to the drug metabolism, lipid peroxidation and the antioxidant nature of α-tocopherol.

Keywords. Doxorubicin; lipid peroxidation; glycolysis; glycogenolysis; vitamin E.

Introduction

Doxorubicin (adriamycin) an antitumor antibiotic is a tetracyclic aglycone to which an amino sugar is attached through a glycosidic linkage (Young *et al.*, 1981). Most of the anthracyclines are metabolised primarily by carbonyl reduction to alcohols (doxorubicinol) which are active metabolites and account for the cytotoxicity of the drug (Huffman *et al.*, 1972).

Based on *in vitro* model of toxicity using isolated hepatocytes and liver microsomes, doxorubicin has been shown to undergo redox cycling between the semiquinone and quinone radicals during its oxidative metabolism. This results in the generation of superoxide anion, hydrogen peroxide and consumption of hepatic and cardiac glutathione during glutathione peroxidase reduction of H_2O_2 (Pollakis *et al.*, 1983). The inherent high risk of developing cardiomyopathy and liver function abnormalities have severely restricted the application of this useful antitumor drug.

Delcers and Goormaghtigh (1985) have demonstrated by an *in vitro* study, that therapeutic doses of doxorubicin are highly toxic to endocrine function mainly on insulin secretion. Since insulin exerts profound effects on the metabolism of glucose and since liver is the major organ involved in the process, an attempt has been made to study the effect of doxorubicin on the levels of certain key enzymes in glucose metabolism, the level of blood glucose and hepatic glycogen content.

Since the free radicals produced during the metabolism of the durg are considered as responsible for the alterations induced in the various cellular macromolecules (Kim *et al.*, 1985), the effect of the antioxidant (Tappel, 1972) α-tocopherol which intercepts the toxic free radicals was also taken for investigation.

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Materials and methods

Chemicals

Doxorubicin hydrochloride, glucose-6-phosphate, glucose-1-phosphate and glycogen were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Analytical grades of anthrone, *p*-hydroxy diphenylamine and sodium pyruvate were obtained from E. Merck, Bombay.

Methods

Weanling, male albino rats derived from Wistar strain, weighing from 90–100 g were selected for the study. The animals were obtained from Department of Forensic Science, Madras. The animals were allowed free access to food and water. The animals were divided into 3 groups. Group I animals served as control. Groups II and III animals received doxorubicin, 2 mg/kg body wt., twice a week intravenously for 6 weeks (Adi *et al.*, 1981). Group III animals were also given α-tocopherol orally, 400 mg/kg body wt. for 2 months.

After the experimental period the animals were killed by cervical decapitation. Fresh blood (0·1 ml) was collected in 10% tricarboxylic acid for glucose estimation (Sasaki *et al.*, 1972).

Liver was dissected out and washed with ice-cold saline. About 100 mg tissue was immediately digested in 30% KOH for glycogen estimation (Morales *et al.*, 1973). One per cent liver homogenate was prepared in 0·1 M Tris buffer, pH 7·5 and this homogenate was used for the estimation of glucose-6-phosphatase (Koide and Oda, 1959), hexokinase (Branstrup *et al.*, 1957), glycogen phosphorylase (Cornblath *et al.*, 1963), lactate dehydrogenase, aldolase (King, 1965) and glucose-6-phosphate dehydrogenase (Eels and Kirkman, 1961). The remaining portion of the liver was used for the separation of mitochondria by differential centrifugation and the activities of isocitrate dehydrogenase (Bell and Baron, 1960), α-Ketoglutarate dehydrogenase (Reed and Mukherjee, 1969), succinate dehydrogenase (Veeger *et al.*, 1969) and malate dehydrogenase (Kitto, 1969) were estimated in the mitochondrial fraction. The protein content was estimated by the method of Lowry *et al.* (1951).

Student's '*t*' test was conducted for the statistical analyses of each parameter.

Results and discussion

The effects of doxorubicin and α-tocopherol on blood glucose level and liver glycogen content are presented in table 1. In doxorubicin treated rats, the blood glucose increases by 20%. This could have been due to the toxic effect of doxorubicin on the pancreatic function as reported by Delcers and Goormaghtigh (1985) in *in vitro* studies. Insulin is a major hormone involved in the maintenance of glucose homeostasis (Lee and Weber, 1968). The hyperglycemia observed in our results might have been due to the lesser availability of insulin. α-Tocopherol co-

Table 1. Effect of doxorubicin and α-tocopherol on the blood sugar level and hepatic glycogen content.

Group	Blood sugar mg/100 ml	Glycogen mg/g liver
I	61·5 ± 1·3	32·5 ± 1·0
II	80·2 ± 2·2[b]	59·2 ± 1·9[a]
III	65·3 ± 1·6[NS]	36·0 ± 2·1[NS]

Values are expressed as mean ± SD from 6 animals in each group.
[a] $P < 0·001$. [b] $P < 0·01$. [NS] Not significant.

ministered animals (group III) show reduced blood sugar level when compared to
ıt of group II animals. Dietary supplementation of α-tocopherol in diabetic rats
; been found to reduce blood sugar level considerably (Beirenbaum *et al.*, 1985).
A significant increase in hepatic glycogen content (table 1) is noted in
xorubicin treated animals which could be attributed to a reduction in the
ivities of glycogen phosphorylase and glucose-6-phosphatase (table 2). Co-

Table 2. Effect of doxorubicin and α-tocopherol on the level of glycogen phosphorylase, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase.

Group	Glycogen phosphorylase (A)	Glucose-6-phosphatase (B)	Glucose-6-phosphate dehydrogenase (C)
I	38·1 ± 0·9	31·2 ± 1·1	3·0 ± 0·1
II	28·5 ± 1·1[a]	20·2 ± 0·8[a]	1·6 ± 0·05[a]
III	35·9 ± 0·85[NS]	27·9 ± 1·0[NS]	2·6 ± 0·001[NS]

Values are expressed as mean ± SD from 6 animals in each group. (A), (B), nmol of P_i liberated/min/mg protein. (C), Units/mg protein.
[a] $P < 0·01$. [NS] Not significant.

ministration of vitamin E prevents this alteration considerably, because of the
ioxidant nature of α-tocopherol against lipid peroxidation induced by
xorubicin (Doroshow, 1983). As the lipid peroxidation products are reported to
ıctivate enzymes (Kim *et al.*, 1985), the alterations induced in these enzyme levels
ght have been through the lipid peroxidative nature of doxorubicin.

The glycolytic enzymes hexokinase, aldolase, lactate dehydrogenase (table 3) are
ınd to be increased in doxorubicin treated rats. Increase in the activity of
ιokinase has been reported in hyperglycemia (Novello *et al.*, 1969).

The concentrations of lactate and pyruvate are presented in table 4. A 20%
rease in both pyruvic and lactic acid concentration in doxorubicin treatment
y be the result of enhanced glycolysis. The lactic acid/pyruvic acid ratio is not
ınd to be altered significantly.

The enzymes involved in the aerobic oxidation of pyruvate in mitochondria,
citrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase
l malate dehydrogenase (table 5) are found to be decreased in doxorubicin
ated animals. The intracellular calcium concentration in mitochondria has been

Table 3. Effect of doxorubicin and α-tocopherol on the level of glycolytic enzymes.

Group	Hexokinase	Aldolase	Lactate dehydrogenase
I	5·10 ± 0·10	152 ± 9·2	1275 ± 41·5
II	8·30 ± 0·190[a]	186 ± 11·3[a]	1690 ± 39·2[a]
III	5·90 ± 0·21[NS]	163 ± 8·9[NS]	1350 ± 50·9[NS]

Values are expressed as mean ± SD from 6 animals in each group. Hexokinase activity is expressed as nmol glucose-6-PO$_4$ formed/min/mg protein. Aldolase activity is expressed as nmol glyceraldehyde-3-PO$_4$/h/mg/protein. Lactate dehydrogenase, nmol of pyruvate formed/min/mg protein.

[a] $P < 0·01$. [NS] Not significant.

Table 4. Effect of doxorubicin and α-tocopherol on the level of lactate and pyruvate content in liver.

Group	Lactic acid (μg/mg protein)	Pyruvic acid (μg/mg protein)	Lactic acid/pyruvic acid ratio
I	3·91 ± 0·1	1·25 ± 0·03	3·128 ± 0·03
II	4·6 ± 0·11[a]	1·50 ± 0·05[a]	3·06 ± 0·05
III	3·51 ± 0·12	1·45 ± 0·02	2·42 ± 0·09

Values are expressed as mean ± SD from 6 animals in each group.

[a] $P < 0·01$.

Table 5. Effect of doxorubicin and α-tocopherol on the level of tricarboxylic acid cycle enzymes.

Group	Isocitrate dehydrogenase	α-Ketoglutarate dehydrogenase	Succinate dehydrogenase	Malate dehydrogenase
I	0·87 ± 0·01	0·45 ± 0·02	0·09 ± 0·001	0·45 ± 0·001
II	0·53 ± 0·015[a]	0·21 ± 0·001[a]	0·051 ± 0·002[a]	0·23 ± 0·009[a]
III	0·79 ± 0·009[NS]	0·39 ± 0·015[NS]	0·079 ± 0·005[NS]	0·38 ± 0·001[NS]

Values are expressed as mean ± SD from 6 animals in each group. Isocitrate dehydrogenase, succinate dehydrogenase and malate dehydrogenase activities are expressed as change in absorbance/min/mg protein. A unit of α-ketoglutarate dehydrogenase is the amount of enzyme required to produce 2 mmol of ferrocyanide/mg protein under incubation conditions.

[a] $P < 0·01$. [NS] Not significant.

reported to be increased during doxorubicin therapy (Crompton and Carafoli, 1979). This intracellular Ca^{2+} is an inducer of phospholipase A$_2$ activity which degrades membrane phospholipids (Porumb and Petrescu, 1986) and the free radicals produced as a result of this lipid peroxidation may attack the protein corresponding to these enzymes. α-Tocopherol treatment prevents the alterations in the above mentioned enzyme levels, probably by counteracting the free radicals or by inhibiting the phospholipase A$_2$ activity as reported by Douglaus *et al.* (1986).

As doxorubicin produces hypoxia (Porumb and Petrescu, 1986), the synthesis of ATP molecules by aerobic oxidation is reduced. To meet the energy demand glycolysis might have been favoured as evidenced from our results (table 3).

Hexose monophosphate shunt pathway is one of the NADPH generating systems and glucose-6-phosphate dehydrogenase activity is found to be decreased in orubicin treated animals (table 2). This result is in accordance with the findings abregat *et al.* (1985). α-Tocopherol treatment enhanced this enzyme activity to a ificant level so that it could provide NADPH for the metabolism of the drug by enzyme Cyt P_{450} reductase and D-T-diaphorase (Sinha *et al.*, 1984) which are DPH-dependent.

rom these results it may be concluded that doxorubicin influences the activities ricarboxylic acid cycle enzymes and glycogenolysis. α-Tocopherol co-administion provides beneficial effects against these alterations. Further studies on the t of insulin and calmoldulin inhibitors are being carried out to find out the hanism of α-tocopherol protection against doxorubicin toxicity.

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pecific staining of myo-inositol-1-phosphatase on polyacrylamide gels fter electrophoresis*

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Abstract. A sensitive staining method was developed to localise the activity of myo-inositol-1-phosphatase on polyacrylamide gels after electrophoresis. The method can also be used for non-specific phosphatases as well as for those specific phosphatases acting upon inositol polyphosphates which are prime cellular second messengers. One or two nmol of phosphate is sufficient and less than 3 μg of purified protein will facilitate the localisation of phosphatase. If more phosphatases are present in the enzyme preparation, a combination of inhibitors can be used to suppress the activities of unwanted phosphatases and the use of specific substrates will facilitate the localisation of enzyme of interest.

Keywords. Myo-inositol-1-phosphatase; alkaline phosphatase; rat testis; levamisole; lithium ions.

ntroduction

The facile localisation of inorganic phosphate (Pi) liberating enzymes after lectrophoresis on polyacrylamide gels is essential to the study of specific hosphatases. The recent identification and characterization of several inositol hosphates specific phosphatases which selectively remove phosphate moieties from nositol ring need a method to localize them on gels after electrophoresis Parthasarathy and Eisenberg, 1986; Berridge, 1987; Majerus *et al.*, 1988; Michell *t al.*, 1989). Although enzyme staining methods on gels have been described for on-specific phosphatases based on the visualisation of a released chromogenic lcohol, these methods have limited value for the detection of phosphatases specific or substrates which generate colourless or non-chromogenic organic fragments. Therefore, it is necessary to detect liberated Pi and methods have been devised in which Pi is variously captured in the gel as a band of white calcium phosphate Alexander, 1968), black lead sulfide (Dvorak and Heppel, 1968), or dye-phospho-molybdate complex (Abrams and Baron, 1967). These methods, however, are uitable for alkaline phosphatases (above pH 10) owing to the increasing solubility f these precipitates at lower pH. The need for a general method grew out of the

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For nomenclature of myo-inositol phosphates recent recommendations are followed. Refer *Biochem. J.*, **58,** 1–2 (1989) and *Eur. J. Biochem.*, **180,** 485–486 (1989). L-myo-inositol-1-phosphate is presently therwise called as D-myo-inositol-3-phosphate.

development of a procedure for purification of myo-inositol-1-phosphatase (EC 3.1.3.25) a specific phosphomonoesterase, optically active at pH 7·7 (Eisenberg and Parthasarathy, 1984) that can hydrolyse both the enantiomeric myo-inositol-1-phosphates originating from the biosynthesis of myo-inositol and the catabolism of phosphatidylinositol (Parthasarathy and Eisenberg, 1986). Having applied the modification (Eisenberg and Parthasarathy, 1984) on the original procedure of Itaya and Ui (1966) for the measurement of Pi, we succeeded in modifying the same technique to stain this enzyme on polyacrylamide gels. Although designed primarily to detect purified phosphatase this method is also suitable for crude system in which specific myo-inositol-1-phosphatase can be distinguished from non-specific alkaline phosphatase by differential inhibition with Li+ and levamisole respectively.

Materials and methods

Materials

Malachite green (oxalate salt), *p*-nitrophenyl phosphate, β-naphthyl acid phosphate, Fast blue RR base, N, N, N′, N′-tetramethyl-ethylenediamine, potato acid phosphatase and poly-1-lysine agarose were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Acrylamide, bisacrylamide, ammonium persulphate, Coomassie brilliant blue R250, and glycine from BioRad, USA. Calf intestinal alkaline phosphatase (1000 U/mg protein) from Miles Laboratories, Illinois, USA. Ammonium molybdate, sodium barbital, and trichloroacetic acid (TCA) from Fisher Scientific Co., Pennyslvania, USA. Rat testicular myo-inositol-1-phosphatase (92 mU/mg protein) was prepared as described elsewhere (Parthasarathy and Eisenberg, 1984). The advantage of this partially purified enzyme is that it is very stable and can be used for several months if kept frozen in aliquots. D-Myo-inositol-1-phosphate dicyclohexyl ammonium salt was prepared from soybean phospholipid by a modified procedure of Ballou (1962). All other chemicals used were of analytical grade. Myo-inositol-1-phosphatase from rat testis was purified to apparent homogenity following 40–60% ammonium sulphate precipitation, ion exchange (Synchropak AX-300) and gel filtration (TSK-3000) type high pressure liquid chromatography, poly-lysine agarose and phenyl sepharose chromatography. Detailed purification procedure will be published elsewhere.

Methods

Polyacrylamide gel electrophoresis (PAGE) was conducted on 6·5 cm gels in 0·5 cm inner diameter × 9 cm pyrex tubes by the method of Davis (1964) as modified by Brewer and Ashworth (1969), except that HCl instead of phosphoric acid was used to adjust the pH in all buffers. Tube gels or thicker (3 mm) slab gels are particularly suitable which retains the colour for longer time.

To stain alkaline phosphatase, gels were removed from their tubes after electrophoresis and transferred to 13 × 100 mm test tubes. The method of Chang and Moog (1972) was followed with slight modification. The incubation medium consisted of 0·125 M sodium barbital, 0·05% sodium β-naphthyl acid phosphate,

% Fast blue RR and 0·003 M $MgCl_2$. Gels were incubated at room temperature
eshly prepared medium until a red band on a colourless background appeared.
bation medium prepared more than 30 min before use will become turbid and
efore unsuitable for staining. To stain by Coomassie blue R-250, gels after
rophoresis were fixed with 12·5% TCA for 1 h and then stained with 0·02% dye
5% methanol-10% acetic acid for 1 h, and destained in 10% acetic acid.

o stain gels by malachite green method, a volume of the solution sufficient to
r tube gel (6 ml) was freshly prepared before use. For non-specific acid
sphatase, 1 ml each of 0·6 M acetate buffer, pH 5, and 0·06 M *p*-nitrophenyl
sphate were added to 4 ml H_2O. For specific myo-inositol-1-phosphatase, 1 ml
of 0·12 M Tris HCl buffer, pH 7·7, 0·018 M $MgCl_2$ and 0·0042 M D-myo-
itol-1-phosphate dicyclohexylammonium salt were added to 3 ml of water.
rnatively L-myo-inositol-1-phosphate (D-myo-inositol-3-phosphate) and 2-
ylic acid can be used as substrates. For non-specific alkaline phosphatase 1 ml
of bicarbonate-carbonate buffer, pH 9·7, 0·018 M $MgCl_2$, 6 × 10–6 M $ZnCl_2$,
0·06 M *p*-nitrophenyl phosphate were added to 2 ml H_2O.

ybdate-malachite green stain

4)6 MoO24·4H_2O (4·2 g) was dissolved in 100 ml of 5 N HCl and 0·15 g malachite
n oxalate was dissolved in 300 ml of water. These two solutions are stable for
ths at room temperature when kept separately. When needed, one volume of
/bdate solution was combined with 3 volumes of malachite greem solution and
30 min it was filtered through Whatman No. 4 paper. The reagent is stable for
it 3 weeks at room temperature but must always be filtered every day just
re use. To stain gels, the filtered reagent was diluted with an equal volume of
. The diluted reagent is tinged green (Eisenberg and Parthasarathy, 1987).
nmediately after electrophoresis, the gel was removed from its tube and then
ersed in the appropriate substrate solution in a 13 × 100 mm test tube and was
bated for 30–45 min at 37°C. For staining acid phosphatase on the gel, a
minary wash in 0·5 M acetate buffer at pH 5 for 10–15 min was given and then
bated with substrate. This wash is necessary to bring the gels to the acid range
h were previously in alkaline range during electrophoresis.

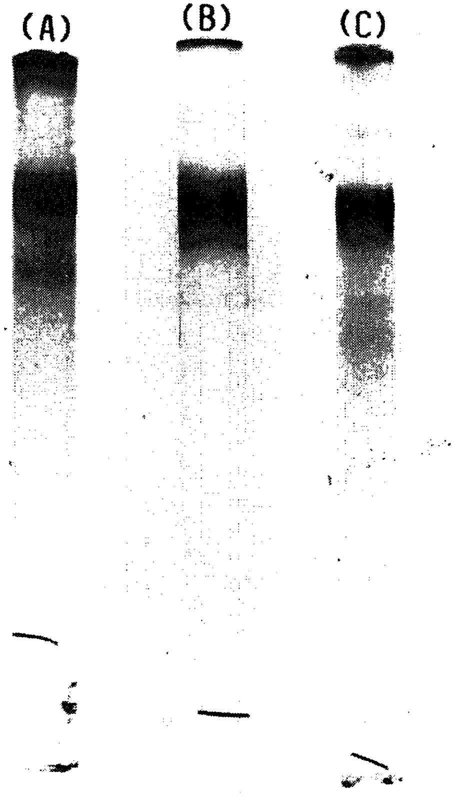
or staining myo-inositol-1-phosphatase, the gels were incubated with diluted
ybdate-malachite green reagent at 37°C for 20–30 min or until the band is
ise. The site of enzyme activity can easily be seen as a distinct green band on a
background. It is preferable to photograph gels as soon as possible. The gels
be stored in 5% acetic acid. This myo-inositol-1-phosphatase activity can be
oited on the gels with 10 mm LiCl.

itivity of detection and amount of protein

method described herein can easily detect Pi in a range 1–2 nmol and less than
of pure protein in the case of inositol-1-phosphatase and less than 1 μg of
line phosphatase is sufficient to localise the site of phosphatase on gels. Figure 1
vs gels stained by the present method and Coomassie blue for rat testicular myo
itol monophosphatase. A series of gels were run with decreasing amounts of

identification. In other methods, sensitivity is lower and takes longer time fo processing (Abrams and Baron, 1967; Alexander, 1968; Dvorak and Heppel, 1968 This method can be applied to the detection of other specific and non-specifi phosphatases. In case of specific phosphatases in crude preparation, the activity ca be visualized by inhibiting the alkaline phosphatase acting by levamisole (1 mM Figure 2 shows the stained gels for alkaline phosphatase by the present method an with naphthyl acid phosphate method (Chang and Moog, 1972). In our experienc acetate buffer is suitable to stain acid phosphatase than citrate buffer.

(1)



(2)

Figures 1 and 2. **1.** Non-denaturing PAGE of myo-inositol-1-phosphatase. (A) The g was stained with Coomassie brilliant blue R250 (5 μg purified enzyme). (B) Specific enzyn stained gel for myo-inositol-1-phosphatase (approximately 2 μg enzyme). **2.** No denaturing PAGE of alkaline phosphatase. (A) The gel was stained with Coomass brilliant blue R250 (5 μg enzyme). (B) The gel stained for phosphatase activity by Fast bl RR method (2 μg enzyme). (C) The gel stained by the present method (2 μg enzyme).

Results and discussion

Our study on the purification and characterisation of myo-inositol-1-phosphata necessitated the development of a method to localise the activity of this enzyme gels and distinguish it from the contaminating phosphatases. After several unsuccessful attempts, we found that a dilute malachite green reagent prepared according to our procedure is capable of staining the enzyme on gels. The method herein, is sensitive, stable and can be performed in less than an hour and can also be used as a general method to localise phosphate liberating enzymes and appropriate buffers should be used for specific phosphatases.

Since the inositol polyphosphates form a pivotal role in second messenger functions, these compounds are generated rapidly in low concentrations and are quickly removed by enzymatic hydrolysis by specific phosphatases (Seyfred *et al.*, 1984; Connolly *et al.*, 1985; Hansen *et al.*, 1987; Erneux *et al.*, 1989). Particularly the last step in the inositol metabolism is carried out by myo-inositol-1-phosphatase, an unique enzyme in the metabolism of inositol phospholipids (Parthasarathy and Eisenberg, 1986). We hope that our present method may be helpful in the task of purification and characterization of various inositol polyphosphate specific phosphatases which control the catabolism of novel second messenger molecules emanating from enhanced turnover of polyphosphoinositides (Michell *et al.*, 1989).

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Transplantation of fetal neocortex in rhesus monkey

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Abstract. A feasibility study of neural transplantation in adult rhesus monkey was undertaken. Fresh and preserved neocortex containing multiplying and maturing neurons obtained from 55–70 gestation days were transplanted into the striatum, cerebellum and cerebral cortex of adult monkeys. Tissues were preserved for 4 days either at subzero temperature in the freezer compartment of the ordinary refrigerator in Ringer lactate or incubated in culture medium. While 2 monkeys out of 5 injected with preserved tissue had successful transplants after 4 months, all the 10 monkeys injected with fresh tissue had no transplants. The size of the two surviving transplants was small. The neurons in the transplants were mainly in clusters. Many of the cells were immature and some showed early degenerative changes. Neuronal processes were restricted to the transplants and thus showed lack of morphological integration with the host tissue. Further studies are in progress to define the nature of the embryonic tissue of primate which can grow and survive and also the role of neural grafts in functional recovery following experimental lesions of the brain regions.

Keywords. Rhesus monkey; fetal neocortex; neural transplantation.

Introduction

Attempts at neural transplantation were already made nearly a century ago. However, successful transplants were obtained only recently with the recognition that embryonic tissue with the potential to proliferate and differentiate was required as donor tissue. As a result, a voluminous literature is now available on neural transplantation in adult mammals, mostly in rodents (Wallace and Das, 1983; Sladek and Gash, 1984; Bjorklund and Stenevi 1985). Till recently hardly any information was available with respect to sub-human primates. Realising this deficiency, two years ago we initiated neural tissue transplantation in rhesus monkeys using embryonic neocortex as the donor tissue, which was at the same stage of development as the donor tissue used in the rat (Gopinath *et al.*, 1987). Based on the report of Rakic (1976) on the sequential development of the neocortex in rhesus monkey, gestation days between 55 and 70 days were selected initially for the donor tissue. Simultaneously the donor tissue was examined to confirm the structural details.

Materials and methods

Cyclicity of the female monkeys was determined by daily examination of the vaginal smear. The regularly cycling females were allowed to mate with the males and the sperm positivity was checked in the vaginal smear the next morning. Accordingly the date of pregnancy was determined. Pregnancy was confirmed by per rectal palpation of the uterine size on 30th and 40th days of the positive vaginal smear. Caesarean sections of the pregnant monkeys were done on the desired dates under aseptic conditions after ketamine anaesthesia (10 mg/kg body weight). Postoperative course of these animals were uneventful and they resumed cyclicity eventually. Neocortex from 4 fetuses of 55, 64, 66 and 70 gestation days were used as donor tissue for transplantation at different sites in the host monkeys (table 1). The crown rump lengths of these fetuses ranged from 4·5–6·5 cm.

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Table 1. Number of host monkeys, sites of transplantation and the nature of donor tissue transplanted and the results.

No. of hosts	Site	Nature of donor tissue	Results
4	Cerebellum	64 days-fresh	Nil
3	Caudate	64 days-subzero temperature	1
2	Caudate	64 days-culture medium	1
2	Cerebellum	66 days-fresh	Nil
3	Motor cortex	55 days-fresh	Nil
1	Motor cortex	70 days-fresh	Nil

Young primates weighing 2–4·5 kg were used as hosts for transplantation. The approximate age ranged between 1 and 2·5 years. Under pentobarbital anaesthesia (35 mg/kg body weight) either small burr-holes were drilled in the skull using stereotaxic coordinates for caudate nucleus, or flaps of the skull were removed to expose the cerebral or cerebellar sites for direct injection of the donor tissue. Just before transplantation the dura was cut to make the injection smooth. Meanwhile the fetus was delivered by caesarean section and the brain was rapidly removed and were immersed in chilled Ringer lactate solution. After removing the meninges and blood vessels, the neocortex from the frontal region was taken into a glass capillary tube attached to a syringe. Solid tissue 50–100 μl was thus injected into the caudate using stereotaxic coordinates in each animal. Neocortex of about 100–200 μl were used for transplantation to the cerebral cortex and cerebellum under direct vision.

Part of the remaining neocortical tissue was preserved in Ringer lactate in the freezer compartment of the ordinary refrigerator or by incubating in culture medium. After 4 days these tissues were transplanted into caudate nucleus using the stereotaxic coordinates. The frozen tissue kept in the freezer compartment of the refrigerator was rapidly thawed by keeping in a warm water bath (50–60°C) before injection. Some of the remaining fresh neocortical tissue was processed for light microscopy to study the stage of development of the donor tissue. Four more fetuses of approximate age group 60–100 gestation days, provided by the Central Drug Institute, Lucknow were also studied to determine the stage of development of the neocortex.

Four months after transplantation two hosts each transplanted in the caudate, sections were cut to identify the transplant if present. The transplants when present with the surrounding host tissue were processed for light microscopy and the sections were stained with cresyl violet for cytoarchitecture and silver stains for fibers.

Table 1 gives the details of the sites and the number of hosts and the age of the donor tissue used in each host for transplantation.

Results

The earliest neocortex studied and transplanted belonged to a 55-day old fetus. Neocortex at this age appeared as a thin shell forming the wall of the lateral ventricle. Under the microscope a ventricular zone, an intermediate zone, a cortical plate and a molecular region were seen. The ventricular zone was a single cell layer of tall cells with a large number of mitotic figures. The cell-rich adjacent area, subventricular layer, showed round immature neuroblasts and spindle shaped migrating cells. The superficial part of this layer had mitotic figures. The remaining region was comparatively free of cells and the ones present were of the migrating type. Subventricular region and the cell-free area between the ventricular layer and the more superficial cortical plate are together termed intermediate zone. Cortical plate was a thick sheet of rounded cells. The marginal zone occupying the subpial position had only very few cells and was very thin (figure 1).

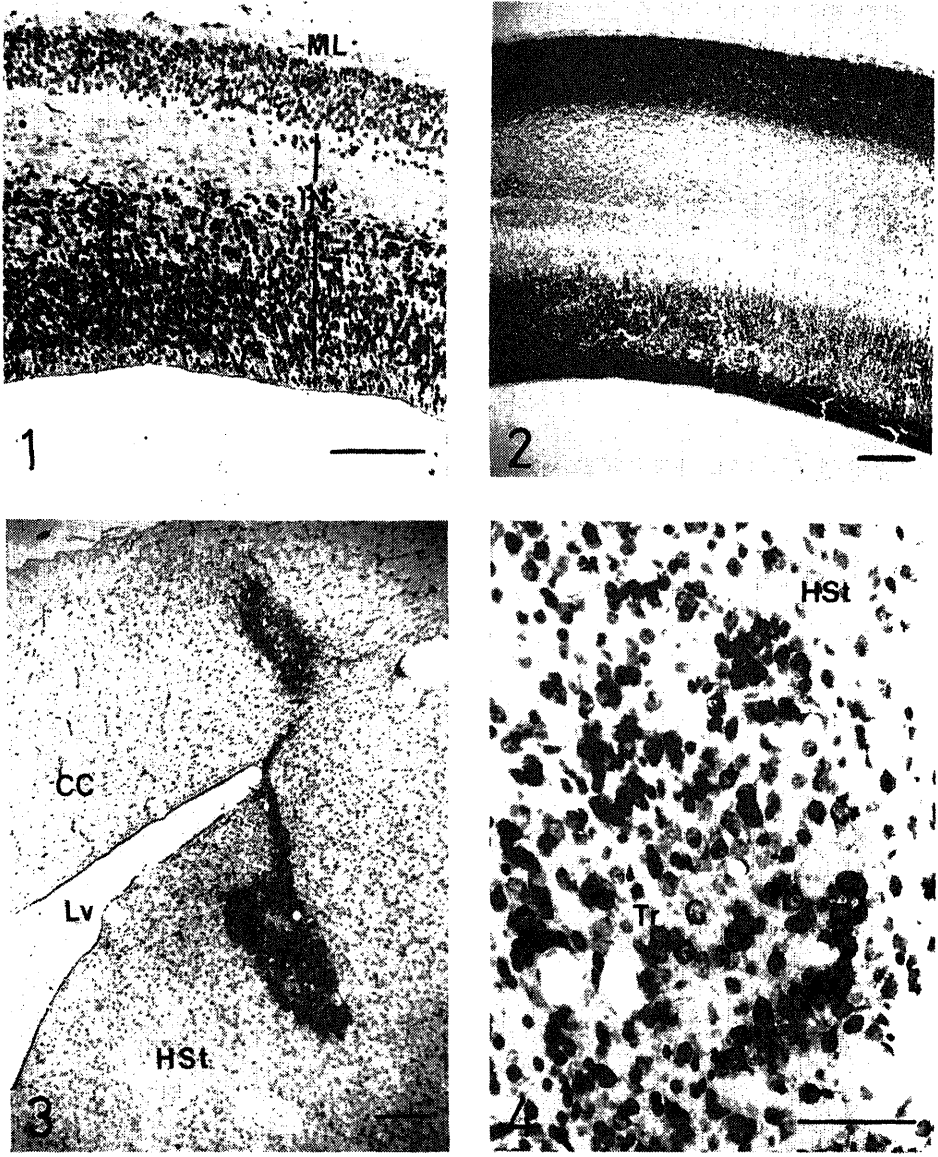
In the older fetuses the neocortex had increased in thickness with the widening of the different layers and differentiation of the cells (figure 2). As the age advanced the number of mitotic figures had declined.

Surprisingly there were no surviving transplants in any of the monkeys in which fresh neocortical tissue was injected. In 4 monkeys, 3 transplanted into cerebellum and one into cerebral cortex, narrow cavities were seen at the injection sites. Host tissue at the margins of these cavities showed destruction and inflammatory cells. In the remaining monkeys, except two injected with preserved donor tissue, injection sites could not be located in serial sections.

In two host monkeys, one injected with tissue maintained in the culture medium and the other with neocortex preserved at subzero temperature, surviving transplants were seen in the striatum. Neocortex used in both the monkeys belonged to gestation day 64. While major part of both transplants remained in the caudate, one transplant extended into the cortex through corpus callosum, showing growth through the needle track (figure 3). Both the transplants were small in size and were well delineated. In both the transplants neurons were mostly in groups and clusters when compared to the scattered distribution of neurons in the surrounding host tissue. Many of the neurons in the clusters had multiple nucleoli suggesting immaturity (figure 4). A few of the scattered neurons had swollen and vesiculated nuclei indicating early degenerative changes. At the margin of the transplants in the surrounding host tissue a few lymphocytes could be seen. The fibers observed in the silver stained preparations were mostly restricted to the transplants and were directed at right angles to the fibre bundles of the surrounding host tissue (figure 5).

Discussion

Studies of Rakic (1976) had already shown that the neocortex of the rhesus monkey



Figures 1–4. **1.** Section of neocortex from 55-day old fetus showing ventricular zone (V), intermediate zone, (IN), cortical plate (CP) and molecular layer (ML). SV, Subventricular layer of the intermediate zone. Bar, 100 μm. **2.** Cross section through the neocortex of 65-day old fetus at low power. The layers seen in figure 1 are still seen but much thicker and better formed. Bar, 200 μm. **3.** Well-circumscribed transplant (Tr) in the striatum (HSt) of the host monkey. Lv, Lateral ventricle. The transplant can be seen extending into the corpus callosum (CC). Bar, 200 μm. **4.** Higher magnification of the transplant (Tr) in the striatum (HSt). Clusters of neurons and neurons with multiple nucleoli can be seen Bar, 50 μm.

grows actively during the period studied by us. In our study the maximum number of proliferating and migrating neurons ideal for grafting as indicated by Das *et al.*

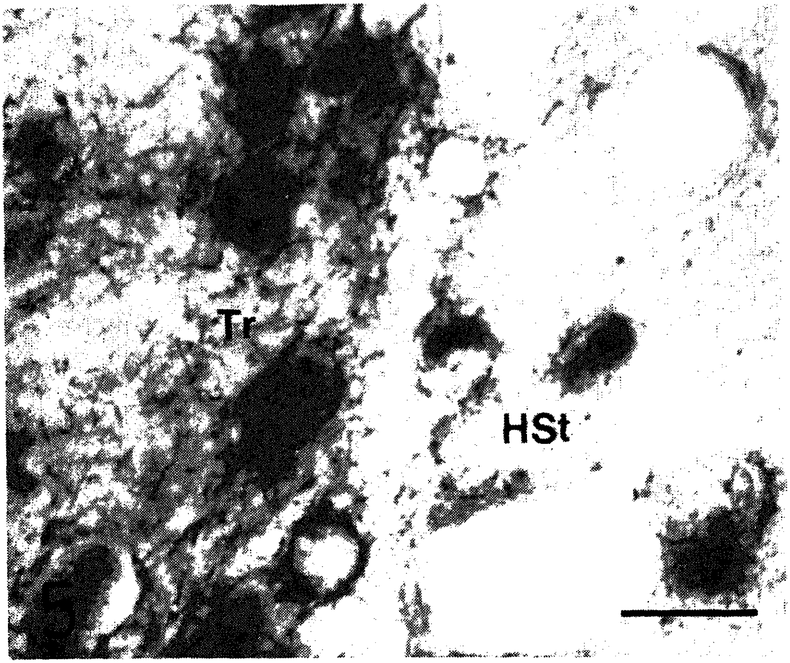


Figure 5. Silver-stained section showing fibres in the transplant (Tr). Fibres of the host striatum (HSt) are not seen as the section is at right angles to the fibre bundles. Bar, 10 μm.

(1980) in rat, is between 50 and 70 gestation days in the somatosensory and motor cortical areas of rhesus monkey. A recent study by Sladek *et al.* (1987) in African green monkeys reported recovery of primates (African green monkeys) from Parkinson-like syndrome induced by 1-methyl-4 phenyl-1,2,3,6-tetrahydropyridine (MPTP) following substantia nigra grafts from advanced gestational days when the nigral cells are already postmitotic (Levitt and Rakic 1982). This success was inspite of the statement by Bakay *et al.* (1987) that successful grafts can only be obtained if the fetal tissue belonged to the 'critical' period of development, which is the stage before the final mitosis. These conflicting reports indicate that for successful transplants in primates dividing cells may not be absolutely essential and that cells undergoing maturation are also as suitable provided the techniques of transplantation and the viability of the donor tissues are properly monitored. From the present study it can be concluded that there were sufficient number of viable neurons after preservation to give rise to surviving grafts at least till the end of the 4th month of transplantation. Houle and Das (1980) have reported that grafts grown from tissues preserved for 6 h at −70°C were comparable to that obtained from fresh donor tissue. Since we have been unsuccessful to obtain even a single surviving graft from the fresh tissue it is difficult to assess the growth potential of the frozen donor tissue in the present study. The presence of immature neurons in the transplant indicate that the maturation of the neurons had been delayed and not comparable to the more advanced maturation of the neurons seen in similar experiments in rat striatum with fresh donor tissue (Gopinath *et al.*, 1987). Structural details of transplants in rhesus monkeys are not available so far for comparison. Limited growth and lack of integration with the host tissue are obvious from the small size and the clear delineation of the transplants. The few lymphocytes in the neighbourhood of the transplants may be a sign of slow rejection. This aspect needs to be investigated.

It is intriguing that while the fresh donor tissue used in 10 monkeys did not take, donor tissue kept either at subzero temperature or in culture medium for 4 days, survived and grew in two out of 5 monkeys. Victorov and Lyjin (1988) having obtained successful transplants in rat from tissues cultivated for 3 h to 9 days, hypothesized that some molecules present in the fresh embryonic tissue may not be conducive to the growth in a different environment. Manasek and Cohen (1977), are also of the view that some molecules of the embryonic tissue may act as inhibitory factors in an alien environment. This view may be supported by the observations that in neonatal hosts as compared to the adults the embryonic donor tissue showed much better growth (McLoon and Lund 1983). The presence of immature neurons and a few showing early degenerative changes either suggest that the adult host tissue is not a conducive environment for the proper maturation and continued maintenance of the transplanted neurons in the primate or that preservation had rendered some of the neurons nonviable.

The present study demonstrated morphologically successful neural transplant in rhesus monkey with donor tissue containing a mixture of proliferating and migrating neurons. Fresh tissue transplanted has failed to grow in this series of study. Inflammatory reaction and cavity at the site of transplantation in a few animals could be due to infection rather than foreign body reaction since such reaction was not present in all. Studies are in progress using other areas of the primate brain as donor tissue to evaluate further the nature of donor tissue ideal for successful transplant and to assess the role of neural grafts in functional recovery in monkeys with experimental lesions of the brain.

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rification and some properties of buffalo spleen cathepsin B

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Abstract. Purification of cathepsin B from buffalo-spleen, a hitherto unstudied system has been achieved by a simple procedure developed by incorporating suitable modifications in the existing methods for isolation of the enzyme from other sources. The purified enzyme has a molecular weight of 25 KDa and its Stokes radius was found to be 2·24 nm. Effects of several reducing agents, urea and thiol-protease inhibitors such as leupeptin and antipain, have been studied and the data unequivocally support the contention that the buffalo-enzyme is similar to cathepsin B from other tissues with respect to these properties.

Keywords. Lysosomal proteases; cathepsin B; buffalo-spleen; hydrodynamic properties.

roduction

hepsin B (EC 3·4·22·1) is the best known and characterized of the numerous osomal cysteine proteinases (Barrett, 1977). The enzyme is considered to play a role in intracellular protein degradation (Huisman *et al.*, 1974; Dean, 1976; nn and Aronson, 1977), modifications of several proteins (Graf and Kennessey, 6; Bond and Barrett, 1980) and several pathological conditions (Poole *et al.*, 8; Stracher *et al.*, 1978; Recklies *et al.*, 1980; Sher *et al.*, 1981; Honn *et al.*, 1982; rnett *et al.*, 1983; Ostensen *et al.*, 1983). The enzyme is also a dipeptidylcarboxy-tidase which releases dipeptides sequentially from the carboxyl ends of poly-tides (Aronson and Barrett, 1978; Nakai *et al.*, 1978; Bond and Barrett, 1979). exact role(s) of cathepsin B *in vivo*, however, still remains speculative.

ne of the major factors that makes the studies on cathepsin B difficult is un-ilability of the pure enzyme in sufficient quantities. Several tissues of limited ilability have been used as the sources for cathepsin B (Barrett and Kirschke, 1) but it is only recently that the purification of the enzyme has been described m tissues easily available in large quantities (Takahashi *et al.*, 1984; Fazili and sim, 1986). In the present study we have chosen buffalo-spleen as the source for hepsin B. A simple procedure for the isolation of the enzyme has been developed l some of its molecular properties are reported.

terials and methods

terials

eens of freshly slaughtered buffaloes were collected from the local slaughter

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reviations used: BANA, Nα-Benzoyl-DL-arginine-β-naphthylamide; DMSO, dimethylsulphoxide; T, dithiothreitol; SDS, sodium dodecyl sulphate; BSA, bovine serum albumin; DTNB, 5,5′-dithio-bis-itrobenzoic acid); PAGE, polyacrylamide gel electrophoresis.

house and were stored at freezing temperature until used. Antipain, Nα-benzoyl-DL-arginine-β-naphthylamide (BANA), dimethylsulfoxide (DMSO), dithiothreitol (DTT), guanidine hydrochloride, 2-mercaptoethanol, sodium dodecyl sulphate (SDS), bovine serum albumin (BSA) (lot 80F-0508), α-chymotrypsinogen (lot 29C-8010), cytochrome *C* (lot 124F-7155), myoglobin (lot 61F-7035), and ovalbumin (lot 23F-8175) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Blue Dextran 2000, DEAE-cellulose and Sephadex G-75 and G-100 were the products of Pharmacia, Sweden. 5,5′-Dithio-bis-(2-nitrobenzoic acid) (DTNB), reduced glutathione and thioglycerol were procured from Sisco, Bombay. All other reagents used were of analytical grade.

Enzyme preparation

The purification of cathepsin B from buffalo spleen was performed by incorporating suitable modifications in the procedure described earlier (Fazili and Qasim, 1986). This modification primarily improved the purity of the enzyme. The soft tissue-mass (2·3 kg) obtained from 3 spleens (gross weight 3·3 kg) was homogenized for 10 min in 1·2 litre of 3% sodium chloride solution containing 1 mM EDTA and 15 mM HCl, and then stirred for 6 h at 4°C. After adjusting the pH of the resulting homogenate to 3·8, it was kept overnight at 4°C. The content was then centrifuged twice at 23,700 *g* for 15 min each at 4°C and the clear supernatant thus obtained was subjected to salt fractionation. A protein fraction precipitating between 40 and 75% $(NH_4)_2SO_4$ was collected and dialyzed against 0·05 M sodium acetate buffer, pH 5, containing 1 mM EDTA and 0·02% sodium azide. It was again centrifuged at 23,700 *g* for 5 min at 4°C and chromatographed on a Sephadex G-75 column (2·6 × 90·0 cm) equilibrated with the above buffer. Enzymatically active fractions were pooled, concentrated and subjected to ion-exchange chromatography on DEAE-cellulose column equilibrated with 0·02 M Tris-acetate buffer, pH 6, containing 1 mM EDTA and 0·02% sodium azide. After washing the column extensively with the above buffer, bound fraction of cathepsin B was eluted stepwise with the same buffer containing 0·2, 0·5, and 1·0 M sodium chloride. Cathepsin B eluted at 0·2 M sodium chloride, was further purified by re-chromatography on the Sephadex G-75 column.

Enzyme assay

The activity of cathepsin B was measured at pH 6·5 and at 37°C using BANA as substrate by the procedure described earlier (Khan *et al.*, 1986). One unit of activity was defined as the amount of enzyme necessary to release 1 μM of 2-naphthylamine per h.

Protein determination

Protein was estimated by the method of Bradford (1976) with BSA as a standard.

Assay of SH-group

ence of 6 M guanidine hydrochloride was determined essentially by the method Ellman (1959). The buffer used was 0·08 M Tris-HCl, pH 8, containing 0·5 mg TA per ml. The reaction was found to be completed within 30 min for the erimental conditions used in this study.

trophoresis

acrylamide gel electrophoresis (PAGE) in the absence and presence of SDS was ormed according to the methods of Davis (1964) and Weber and Osborn (1969) ectively. The cross-linking of the gel in both the experiments was 10%.

rescence measurements

rescence was measured with 1 cm cell at 37°C with a Shimadzu RF 540, trofluorophotometer. The excitation and emission wavelengths were 335 and nm respectively for the measurement of the enzyme activity (Khan *et al.*, 1986).

filtration on Sephadex G-100

e hydrodynamic constants were calculated from the data obtained by gel ation on a Sephadex G-100 column (2·6 × 88·0 cm) equilibrated with 0·06 M um phosphate buffer, pH 6·5. The elution volume of the Blue Dextran 2000 was n as the void volume (V_o), and the total volume (V_t) was determined from the on volume of $K_3Fe(CN)_6$. The value of frictional ratio (f/f_o) was obtained by procedure described in Fazili and Qasim (1986) using 0·742 as the value of ial specific volume.

ct of various compounds

effects of urea, antipain, leupeptin and various reducing agents on the activity athepsin B were studied by measuring the activity in usual manner except that enzyme was incubated with the described compound for 30 min prior to assay s activity.

ults and discussion

fication of buffalo-spleen cathepsin B

mpts to utilise previously published procedures for the isolation and purification athepsin B yielded a heterogeneous product as judged by PAGE (Otto, 1971; li and Qasim, 1986). We, therefore, experimented with various combinations of iltration and ion-exchange chromatography to isolate cathepsin B from buffalo-en. The purification steps are summarized in table 1. When the ammonium hate precipitated protein at step 3 of table 1 was applied to a Sephadex G-75 mn (2·6 × 90·0 cm) and eluted with 0·05 M sodium acetate buffer, pH 5, con-

Table 1. Purification of buffalo-spleen cathepsin B.

Purification steps	Total protein (mg)	Total enzyme activity (units)	Specific activity (units/mg)	Yield (%)	Fold-purification
Crude extract	148000	31620	0·214	100	1
Acid extraction	6225	17413	2·784	55	13
Ammonium sulphate fractionation	930	8316	8·932	26	42
Sephadex G-75 chromatography	271	3330	12·293	11	57
DEAE-cellulose chromatography	25·4	492	19·395	1·6	91
Sephadex G-75 re-chromatography	9·38	219	23·324	0·7	109

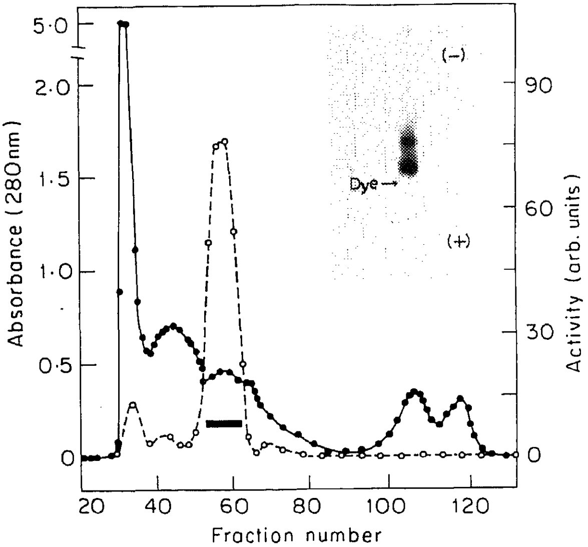

Figure 1. Sephadex gel chromatography of $(NH_4)_2SO_4$ fraction (40–75%) of buffalo-spleen cathepsin B on Sephadex G-75 column (2·6 × 90·0 cm) equilibrated with 0·05 M sodium acetate buffer, pH 5, containing 1 mM EDTA and 0·02% sodium azide. The fractions (5 ml) were read at 280 nm for protein (●), and monitored for BANA hydrolase activity (○). Horizontal bar indicate the fractions pooled for further purification. Inset shows the PAGE pattern of the purified enzyme in 10% gel using 0·02 M Tris-glycine buffer, pH 8·3.

by horizontal bar in figure 1) containing significant amount of BANA hydrolase activity were combined and subjected to ion-exchange chromatography on DEAE-cellulose column. The cathepsin B obtained at this stage was further purified by re-chromatography on Sephadex G-75 column to give the final enzyme preparation. The product of this purification method was found to be homogeneous with respect to size as it gave a single symmetrical protein peak fully superimposable with its activity peak on Sephadex G-100 column (2·6 × 88·0 cm). PAGE of the enzyme on 10% gels at pH 8·3 (figure 1, inset), resulted into two bands. Four minor bands ported by Fazili and Qasim (1986) were not observed under any electrophoretic nditions used by us. Protein fractions corresponding to the two bands, esumably isozymes of cathepsin B (Otto and Reisinkonig, 1975; Takahashi *et al.*, 86), were however, not further separated.

This procedure was very efficient in terms of time, labour, total enzyme units and ecific activity but inefficient in terms of yield and fold-purification (table 1). This is t unusual since wide variations in the degree of purification of cathepsin B have en documented (Otto and Reisinkonig, 1975; Hirao *et al.*, 1984; Takahashi *et al.*, 86). A lower degree of purification (109-fold) obtained does not necessarily dicate the presence of impurities in our preparation since fold-purification also pends, among others, on the source of the enzyme and on the substrate used for assay (Fazili and Qasim, 1986). A close examination of table 1 does indicate that atively high specific activity obtained in the crude extract of buffalo-spleen is a aracteristic feature of this tissue that might affect the final fold-purification.

aracteristics of buffalo-spleen cathepsin B

e chemical and enzymatic properties of purified enzyme were similar to those scribed for cathepsin B from other sources (Barrett, 1977). Under native conditions where no exogeneous thiols were added, the enzyme was found to contain out 0·3 mol of SH-group per mol of the protein. When the estimation of thiol oups was performed in the presence of 6 M guanidine hydrochloride, the SH-oup content increased to about 1 mol per mol of the enzyme. The molecular ight of the enzyme, as obtained by SDS–PAGE, was found to be 23 KDa.

As can be seen in table 2, various reducing agents have very strong stimulatory ect on the enzyme-activity. These results suggest that buffalo-spleen cathepsin B a cysteine protease that requires thiol reducing compounds for its activity. Effects

Table 2. Effect of various thiol-reducing compounds on the activity of buffalo-spleen cathepsin B.

Concentration (mM)	Enzyme activity (%)				
	2-Mercaptoethanol	DTT	L-Cysteine	Glutathione	Thioglycerol
0	3	2	2	2	3
1	52	90	83	60	77
2	62	100	94	77	94
3	64	96	94	85	120
4	73	88	93	68	128
6	76	80	74	68	128
8	76	79	43	51	128
10	78	74	16	26	128

Enzyme activity obtained in the presence of 2 mM DTT is taken as 100%. To 0·1 ml (0·181 mg) of the enzyme solution was added 1·9 ml of an activator solution in 0·02 M sodium phosphate buffer, pH 6·5, containing 2 mM EDTA and various concentrations of thiol-reducing compounds. After incubating the contents at 37°C for 30 min, 1 ml BANA solution (0·1%) was added and the hydrolase activity was measured fluorometrically (Khan *et al.*, 1986). The values are average of two determinations.

of leupeptin and antipain on the activity of buffalo-spleen cathepsin B were studied at different inhibitor concentration and the results are depicted in figure 2 which also include the data on the influence of urea on the protease activity (figure 2, inset). It is evident from the figure that almost all the activity is lost at inhibitor concentrations of 0·01 mM and above. Out of the two inhibitors, leupeptin appears to be more effective. Buffalo-spleen cathepsin B was also found to be very sensitive towards urea (figure 2, inset). The enzyme was readily inhibited by more than 40% at urea concentrations as low as 0·1 M. The degree of inhibition was about 80% at 0·5 M urea and practically no activity was left at urea concentrations of 1 M and above. This is not in full agreement with the recent report (Fazili and Qasim, 1986) on buffalo cathepsin B from another tissue, namely liver, where 3 M urea was required to abolish the enzyme activity by more than 90%.

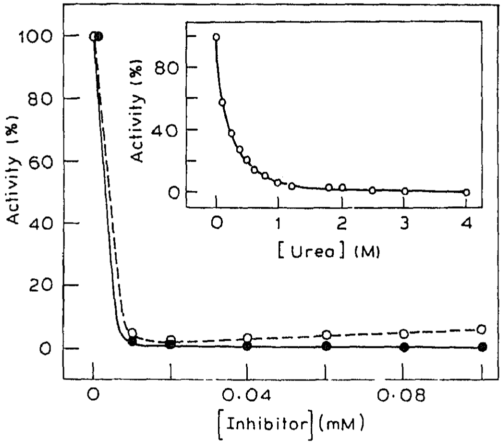

Figure 2. Influence of leupeptin (●) and antipain (○) on the activity of buffalo-spleen cathepsin B. Inset shows the dependence of the catheptic activity on urea concentration. Enzyme activity in the absence of the inhibitors was taken as 100%. A 0·02 M sodium phosphate buffer (pH 6·5) containing 2 mM each of EDTA and 2-mercaptoethanol was used as activator. Other details were the same as described in table 2.

Above data on dependence of the activity of the buffalo-spleen enzyme on various reducing agents and inactivators are not only in agreement with the earlier reports on cathepsin B from other sources (Barrett, 1977; Bradley and Whitaker, 1986) but also rule out the presence of cathepsins H and L in our preparation. Since cathepsin H shows little sensitivity towards leupeptin, and urea has stimulatory effects on cathepsin L activity (Barrett and Kirschke, 1981), BANA hydrolase activity of our enzyme preparation, resolved into the two bands by PAGE (figure 1, inset), can only be attributed to the two isozymes of cathepsin B (Otto and Reisinkonig, 1975; Takahashi *et al.*, 1986).

e data by the methods of Porath (1963) and Laurent and Killander (1964) ed straight lines (shown in figure 3) following the equations:

$$M^{1/3} = 86{\cdot}205 - 70{\cdot}274\, K_d^{1/3} \tag{1}$$

$$\text{and} \quad (-\log K_{av})^{1/2} = 0{\cdot}1837\, r + 0{\cdot}1383, \tag{2}$$

e M and r were the molecular weight and Stokes radius and were computed above equations to be 25 KDa and 2·24 nm respectively. K_d and K_{av} represent bution coefficient and available distribution coefficient, respectively. The value ctional ratio corresponding to these values of molecular weight and Stokes s was calculated by the method described by Fazili and Qasim (1986), to be Computation of the geometric mean radius by the method of Ackers (1970) as ibed by Suelter (1985) yielded a value of 1·86 nm.

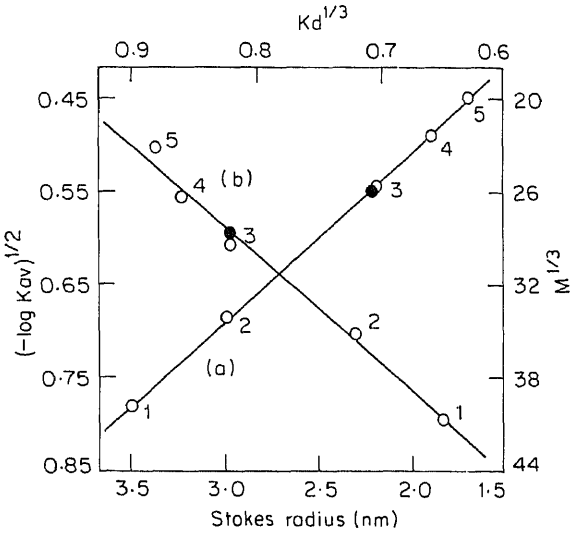

Figure 3. Treatment of gel filtration (data) (a) for the determination of Stokes radius according to Laurent and Killander (1964), and (b) for the determination of molecular weight according to Porath (1963). Straight lines (a) and (b) were drawn by the method of least squares and represent plots $(-\log K_{av})^{1/2}$ *versus* Stokes radius and $K_d{}^{1/3}$ *versus* $M^{1/3}$, respectively. The marker proteins used were: 1, BSA; 2, ovalbumin; 3, α-chymotrypsinogen; 4, myoglobin; and 5, cytochrome *C*. The positions of cathepsin B are indicated by filled circles.

ese results suggest that although the enzyme molecule is considerably hydrated es radius is significantly higher than geometric mean radius), it exists in a act and globular conformation since its frictional ratio is close to 1 (Yang,). Furthermore, the fact that the value of molecular weight obtained by SDS– E is lower (by about 2 KDa) than the value calculated from the gel filtration viour of the enzyme also indicate that cathepsin B is excessively hydrated r its native conditions. This should not be surprising for cathepsin B is known ntain significant amount of carbohydrate (Takahashi *et al.*, 1984; Sarfraz ad and M. Yahiya Khan, unpublished results).

Acknowledgements

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A rapid procedure for the isolation of lysosomes from kidney cortex by Percoll density gradient centrifugation

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Abstract. Highly pure lysosomes were isolated from buffalo (*Bubalus bubalis*) kidney cortex by a procedure involving differential and isopycnic Percoll density gradient centrifugations. Arylsulphatase, N-acetyl-β-glucosamindase and cathepsin D in the lysosomal preparation were 26–45-fold enriched over the homogenate. The purified lysosomes contained less than 0·06% of mitochondrial, microsomal and peroxisomal marker enzymes. In the electron micrographs the particles appeared as large dense granules of size 0·3–1·9 μm with no apparent structural features belonging to mitochondria or microsomes. The isolation procedure was also found to be suitable to obtain highly pure lysosome particles from renal cortex of other sources such as rat, lamb and beef. No ultracentrifugation steps were involved in the procedure.

Keywords. Lysosomes; kidney cortex; Percoll gradient; isolation procedure; buffalo kidney.

Introduction

Lysosomes are recognized as an important site of intracellular catabolism (Gordon, 1973; Pontremoli *et al.*, 1984; Harikumar and Ninjoor, 1985). Isolation of these organelles in a highly pure form is an essential step in establishing their structure-function relationships. However, separation of lysosomes free from other subcellular contaminants has proved difficult mainly because their size and equilibration densities overlap considerably with that of mitochondria and microsomes (Beaufay, 1972). Most of the successful attempts at the isolation of lysosomes, therefore, involve the use of density altering agents (Leighton *et al.*, 1968; Beaufay, 1972; Arborgh *et al.*, 1974; Harikumar and Ninjoor, 1986), which often adversely affect the permeability characteristics of lysosomal membrane. However, currently available methods employing density gradient media such as metrizamide (Wattiaux *et al.*, 1978) and Percoll (Pertoft *et al.*, 1978; Yamada *et al.*, 1984) have made it possible to isolate pure lysosomes in unmodified form from organ tissues. Harikumar and Reeves (1983) demonstrated that highly enriched lysosomal preparations with minimum contamination of mitochondrial or microsomal marker enzymes could be obtained from rat kidney cortex by Percoll density gradient centrifugation. The present communication reports a rapid centrifugation procedure for the isolation of pure lysosomes from buffalo kidney cortex using Percoll gradient and indicates the efficiency of this method in the isolation of renal lysosomes of high purity from other sources such as rat, beef and lamb.

Abbreviations used: HL, Heavy lysosomes; RSA, relative specific activities.

Glucose-6-phosphate, 5′-adenosine-monophosphate, *p*-nitrocatechol sulphate, *p*-nitrophenyl-N-acetyl-β-D-glucosaminide, haemoglobin type II, *p*-nitrotetrazolium violet and Percoll were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Density marker beads were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade quality. Kidneys of buffalo, lamb and beef were obtained fresh from the local abattoir immediately after slaughter and brought to the laboratory in chilled 0·3 M sucrose containing 1 mM EDTA adjusted to pH 7 with Tris (sucrose buffer). Rat kidney was obtained from male albino rats of Wistar strain weighing 300–400 g. Animals were anaesthetized with ether and kidneys were rapidly excised and chilled in sucrose buffer. Cortex tissues from the kidneys were removed with a razor blade, weighed, minced and rinsed thrice with excess sucrose buffer. This rinsing step is recommended to prevent contamination of the lysosomal preparation with erythrocytes.

Tissue homogenization

All homogenates were prepared in sucrose buffer. Rat kidney cortex was homogenized with 3 up and down strokes in Potter Elvehjem glass homogenizer while kidneys of buffalo, lamb and beef were homogenized with two, 10 s bursts at speed 4 in a polytron tissue homogenizer. The volume of the homogenized tissue suspensions were made up to 10 ml/g tissue with sucrose buffer and then filtered through a single layer of cheese cloth.

Differential and isopycnic density gradient centrifugation

Two of the important physical properties of kidney cortex lysosomes observed earlier (Straus, 1954) are their characteristic grey colour and sticky nature which enabled their visible differentiation from nonlysosomal constituents during the present tissue fractionation. The isolation procedure is schematically represented in figure 1. All centrifugations were carried out in transparent polycarbonate tubes using SS-34 fixed angle rotor fitted to a Sorvall RC-2 refrigerated centrifuge. Homogenates were spun at 120 *g* for 5 min. The sediment was discarded and the supernatant was centrifuged at 12,000 *g* for 20 min. The resulting sediment consisted of a dark grey zone of lysosomes overlaid with a whitish zone which predominantly consisted of mitochondria. The supernatant along with a greater part of the whitish zone was removed by aspiration. Swirling the pellet with sucrose buffer facilitated the removal of the whitish zone while the grey pellet remained undisturbed by this procedure. The pellet, which now consisted largely of the sticky dark grey zone was resuspended in a minimum volume of sucrose buffer to obtain crude lysosome preparation.

Percoll density gradient centrifugation was employed for the further fractionation of crude lysosome. Gradient was prepared by mixing Percoll, 2 M sucrose and 100 mM MOPS/Tris pH 7 in the ratio 14 : 3 : 3 by volume. About 5 ml of the crude lysosome (10–15 mg protein/ml) was mixed with 35 ml of the Percoll mixture and centrifuged at 27,000 *g* for 90 min. After centrifugation 5 distinct zones could be

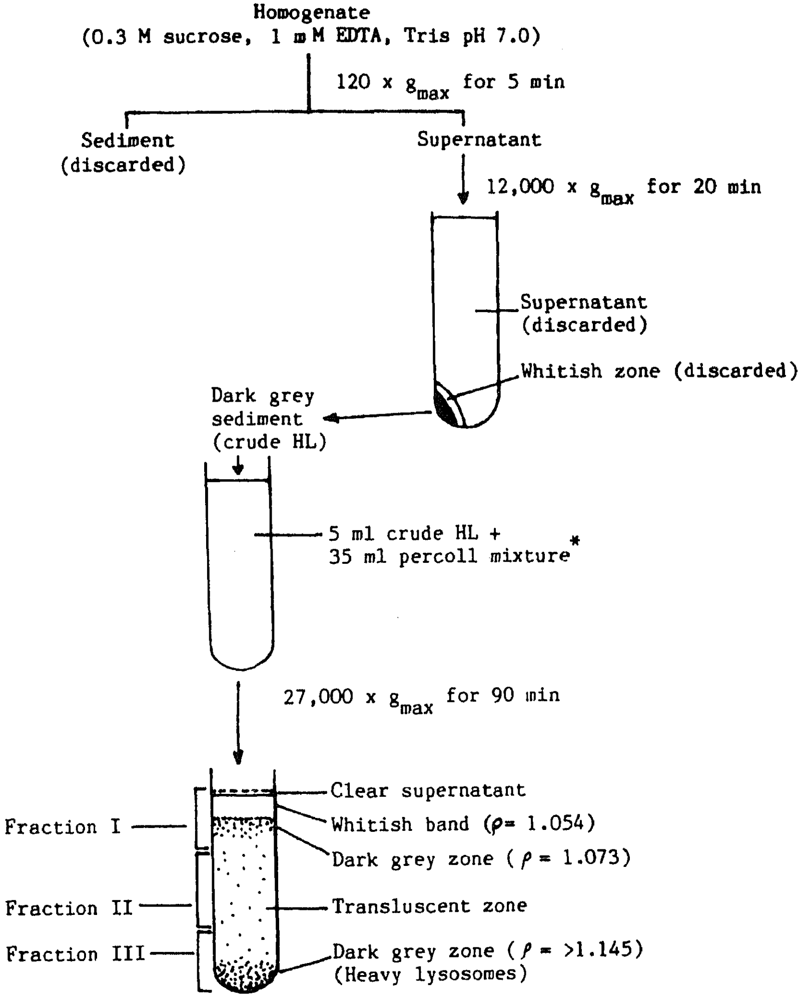

Homogenate
(0.3 M sucrose, 1 mM EDTA, Tris pH 7.0)
120 x g_max for 5 min
Sediment (discarded)
Supernatant
12,000 x g_max for 20 min
Supernatant (discarded)
Whitish zone (discarded)
Dark grey sediment (crude HL)
5 ml crude HL + 35 ml percoll mixture*
27,000 x g_max for 90 min
Clear supernatant
Whitish band (ρ= 1.054)
Dark grey zone (ρ = 1.073)
Transluscent zone
Dark grey zone (ρ = >1.145) (Heavy lysosomes)
Fraction I
Fraction II
Fraction III

Figure 1. Isolation of lysosomes from kidney cortex. All centrifugations were carried out in RC-2 refrigerated centrifuge.
*The Percoll mixture consisted of Percoll, 2 M sucrose and 100 mM MOPS/Tris pH 7 in the ratio 14:3:3 by volume. Unit of density (ρ) is g × cm^{-3}.

rved (figure 1) which, from the top to the bottom of the tube were as follows: clear supernatant; (ii) a whitish band (predominantly mitochondria) librating at $\rho = 1{\cdot}054$ g × cm^{-3}; (iii) a dark grey band (light lysosome) positioned $= 1{\cdot}073$ g × cm^{-3}; (iv) a transluscent zone and (v) another dark grey band vy lysosome, HL) equilibrating at $\rho = 1{\cdot}145$ g × cm^{-3}. These zones were fully separated into 3 fractions (fractions I–III) as outlined in figure 1 by ration from the top of the gradient.

ercoll was removed from the fractions by repeated washing with sucrose buffer. fraction mixed with about 10-fold excess of sucrose buffer was centrifuged at)0 *g* for 10 min. This procedure enabled the sedimentation of particulate

HL from buffalo kidney cortex were fixed with 1% osmium tetroxide in 100 mM sodium cacodylate buffer, pH 7·4, at 0–4°C for 1 h, washed with 100 mM cacodylate buffer, pH 7·4, twice and then pelletted by centrifuging at 1000 *g* for 10 min. Specimens were dehydrated in ethanol and embedded in araldite. Thin sections were cut on a Sorvall MT 5000 ultramicrotome and stained with uranyl acetate followed by lead citrate. Samples were examined with a Zeiss 105 electron microscope.

Enzyme assays

Lysosomal marker enzymes, arylsulphatase (EC 3·1·6·1), N-acetyl-β-D-glucosaminidase (EC 3·2·1·30) and cathepsin D (EC 3·4·4·23) were assayed as described by Barrett and Heath (1977). Activities of 5′-nucleotidase, succinate-*p*-iodonitroletrazolium violet reductase and glucose-6-phosphatase were determined according to Morre (1971) as markers for plasma membrane, mitochondria and microsomes respectively. Catalase (Baudhuin *et al.*, 1964) was assayed as marker enzyme for peroxisomes.

Protein

Protein contents of the samples were assessed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Density

Density distribution in the Percoll gradient was determined by using density marker beads.

Results

As outlined in figure 1, the procedure for the isolation of kidney lysosomes consisted of obtaining a crude fraction from the tissue homogenate by differential centrifugation which was further fractionated by isopycnic banding on Percoll density gradient. The crude lysosome fraction was 2–4-fold enriched in lysosomal marker enzymes over the homogenate and it accounted for 15–20% of arylsulphatase, 10–14% of succinate-INT-reductase, 1–2% of glucose-6-phosphatase and 2–3% of 5′-nucleotidase (data not shown). The distribution pattern of marker enzymes in the 3 different fractions obtained during further fractionation of the crude lysosome on Percoll density gradient is depicted in figure 2. Major part of the lysosomal enzymes (cathepsin D, arylsulphatase and N-acetyl-β-glucosamindase)

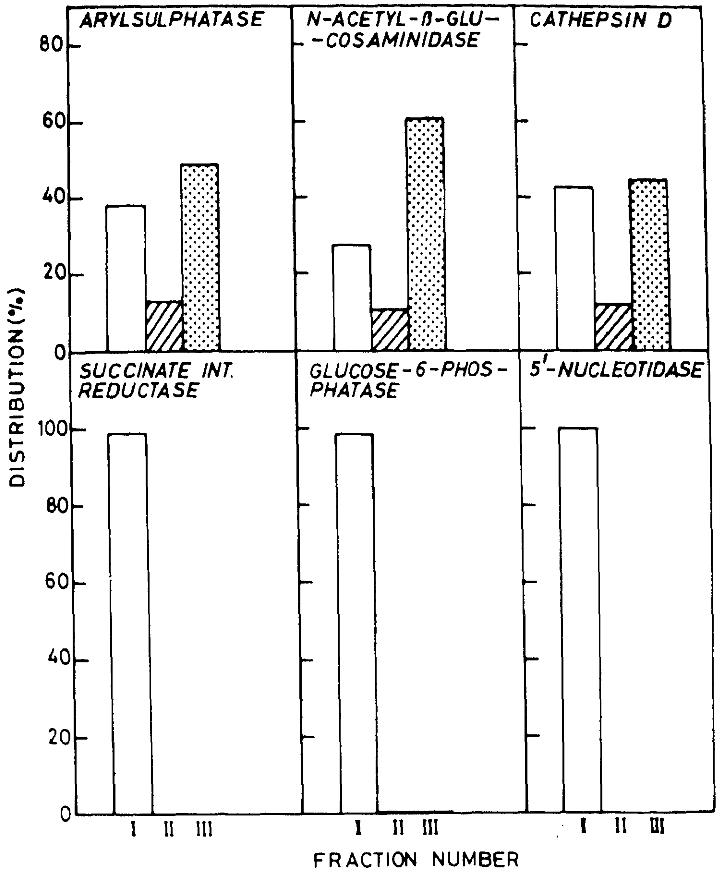


Figure 2. Fractionation of crude HL from buffalo kidney cortex on Percoll density gradient. Crude lysosome fractions isolated from buffalo kidney cortex were mixed with the Percoll mixture and centrifuged at 27,000 *g* for 90 min (materials and methods). Fractions I–III were collected from the top of the gradient by aspiration as outlined in figure 1.

as found to be associated with fraction I (28–38%) and fraction III (45–61%). owever, fraction I also contained a large proportion (99·5–100%) of nonlysosomal arkers such as succinate-INT-reductase, glucose-6-phosphatase and 5'-nucleodase. Apparently fraction I contained light lysosomes ($\rho = 1{\cdot}073$), heavily ontaminated with nonlysosomal constitutents which possess closely similar quilibration densities (figure 1). Fraction III (HL), on the other hand possessed less han 0·003% and 0·06% of succinate-INT-reductase and glucose-6-phosphatase espectively, while 5'-nucleotidase was not detectable. Thus, fraction III apparently onsisted only of lysosomes with little contamination from other subcellular omponents. Data on the purity of lysosomes in this fraction (HL) isolated from uffalo kidney cortex is presented in table 1. Relative specific activities (RSA) for the

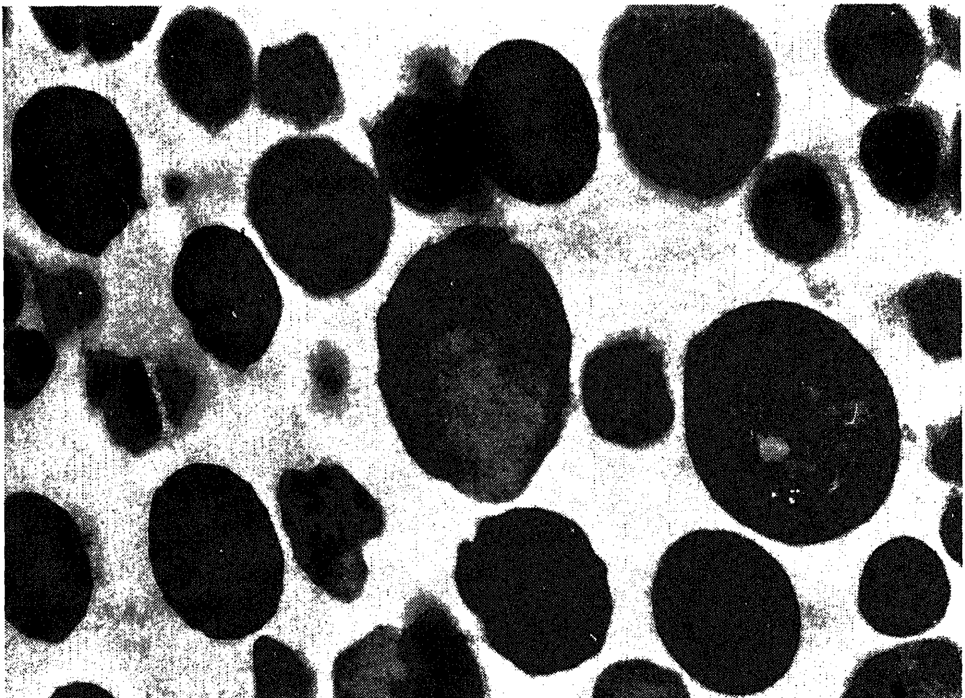
Table 1. Purity of buffalo kidney lysosomes*.

Marker enzymes	n	Specific activity	RSA	Yield (% of homogenate)
Arylsulphatase	9	0·28 ± 0·04	29	3·2 ± 0·8
N-Acetyl-β-D-glucosaminidase	5	1·20 ± 0·30	45	4·8 ± 2·2
Cathepsin D	5	0·30 ± 0·09	26	2·4 ± 1·2
Succinate-INT-reductase	9	0·27 ± 0·27	0·02	0·007 ± 0·007
Glucose-6-phosphatase	9	0·006 ± 0·003	0·04	0·05 ± 0·04
5′-Nucleotidase	9	ND	ND	ND
Catalase	5	0·03 ± 0·01	0·03	0·005 ± 0·001

*Activities of all the enzymes were determined in the presence of 0·1% Triton X-100. Specific activity of catalase represents Δ absorbance at 410 nm/mg protein/min while the activities of all the other enzymes are expressed as μmol/mg protein/min. The values are averages (±SD) of 'n' separate lysosomal preparations. Relative specific activity is calculated as the ratio of the specific activity in the fraction to that in the homogenate. ND, Not detectable.

observations (figure 3) showed lysosomes in HL, as dark dense granules with diameters ranging from 0·3–1·9 μm. The preparation consisted almost exclusively of electron-dense granules. No mitochondrial profile was noticed in 3 different lysosome preparations.

Data on the relative enrichment of lysosomal marker enzymes in HL fractions



ed from beef, lamb and rat kidney cortex homogenates are presented in 2. Relative specific activities of arylsulphatase were 63, 28 and 31 for omes obtained from rat, beef and lamb respectively. The yield of succinate-reductase was less than 0·009% in these preparations. Activities of glucose-6-hatase in rat, lamb and beef lysosomes were 0·18, 0·01 and 0·004% ctively. 5′-Nucleotidase was not detected in lamb lysosomes while its activities and beef respectively were 0·19 and 0·03%.

Table 2. Purity of lysosomes isolated from kidney cortex of rat, beef and lamb*.

	Source		
	Rat	Beef	Lamb
Arylsulphatase			
Specific activities	1·06 ± 0·08	0·19 ± 0·02	0·12 ± 0·01
RSA	63	28	31
Yield (%)	3·7 ± 1·0	0·81 ± 0·6	1·0 ± 0·6
N-Acetyl-β-glucosaminidase			
Specific activities	2·4 ± 0·6	0·95 ± 0·3	0·5 ± 0·1
RSA	57	22	31
Yield (%)	3·2 ± 1·2	0·48 ± 0·2	0·7 ± 0·5
Succinate-INT-reductase			
Specific activities	0·4 ± 0·2	0·11 ± 0·07	0·16 ± 0·07
RSA	0·03	0·007	0·02
Yield (%)	0·009 ± 0·004	0·0003 ± 0·0002	0·001 ± 0·001
Glucose-6-phosphatase			
Specific activities	0·06 ± 0·04	0·013 ± 0·01	0·03 ± 0·006
RSA	0·7	0·06	0·3
Yield (%)	0·18 ± 0·09	0·004 ± 0·003	0·014 ± 0·004
5′-Nucleotidase			
Specific activities	0·03 ± 0·02	0·003 ± 0·001	ND
RSA	0·4	0·3	—
Yield (%)	0·19 ± 0·03	0·03 ± 0·02	—

*Specific activities of all the enzymes are expressed as µmol/mg protein/min.
RSA is calculated as the ratio of specific activity in the fraction to that in the homogenate.
Yield is computed on the basis of the activity in homogenate which is considered as 100%.
ND, Not detected.

ussion

osomes from kidney cortex were first isolated as droplets of varying sizes by us (1954, 1956) using differential centrifugation. Shibko and Tappel (1965) and nsbach (1974) achieved enhanced enrichment of kidney lysosomes by bining the differential centrifugation procedure with sucrose density gradient s. The method described here employs isoosmotic gradient of Percoll to rate lysosomes free of other subcellular contaminants. The RSA of 26–45-fold rved for arylsulphatase, N-acetyl-β-glucosaminidase and cathepsin D (table 1) in buffalo kidney lysosomes are comparable to the corresponding values reported for highly purified lysosome preparations from hepatic (Symons and Jonas, 1987; Nakabayashi and Ikezawa, 1988; Rupar and Whitehall, 1988) and renal tissues (Andersen *et al.*, 1987; Kojima *et al.*, 1987). The purity of buffalo kidney lysosomes is apparent from the low activities of mitochondrial, microsomal and peroxisomal marker enzymes in the preparation (table 1). The absence of nonlysosomal structures in the electron micrograph (figure 3) confirms this observation.Values for structure-linked latency of lysosomal enzymes were found to be in the range of 78–90% (data not shown). This, along with the morphological data (figure 3) depicting the particles as electron-dense granules surrounded by intact membranes points to the integrity of lysosomes. Thus, renal lysosomes obtained by this procedure should be suitable to investigate the permeability characteristics of lysosomal membrane. The earlier investigations of Harikumar and Reeves (1983, 1986) showed that rat kidney lysosomes isolated by the Percoll gradient procedure indeed hold ion gradients with high efficiency.

The efficacy of Percoll method for the successful isolation of renal lysosomes from a variety of sources such as rat, beef and lamb is evidenced by the data presented in table 2. Lysosomal preparations obtained from all these sources showed high enrichment (22–63-fold) of acid hydrolases with little contamination from other subcellular organelles. However, the yield of lysosomes from individual sources was found to vary markedly (table 2). Whether this represented a difference in the content of lysosome particles *in vivo* or it was due to differences in the relative amenability of the tissues to the fractionation procedure is not clear. Since homogenization methods have a decisive role in determining lysosomal integrity (de Duve, 1971; Beaufay 1972), it would be reasonable to assume that the higher yield of lysosomes in rat kidney could at least partly be due to the relatively milder homogenization methods with which this tissue could be disrupted. Despite variations in the yield, the relative specific activities (tables 1 and 2) and structural latency (data not shown) of acid hydrolases in lysosomes isolated from buffalo, rat, beef and lamb (tables 1 and 2) were comparable. Thus, it is evident that the procedure outlined in figure 1 could be used as a general method to obtain highly pure lysosomes from kidney cortex tissue. Four major advantages of the procedure are (i) no density altering agents such as Triton WR-1339 which induce nonphysiological alterations in lysosomes (Leighton *et al.*, 1968; Trout *et al.*, 1981) are used, (ii) the use of Percoll gradients precludes osmotic stress that sucrose would exert on lysosomes, (iii) the method does not involve any ultracentrifugation steps, thus minimizing possible constraints imposed on the organelles by the centrifugal force, and (iv) starting with the cortex tissue, the whole isolation procedure could be completed in less than 3 h. The rapidity of the isolation procedure is particularly important in that, it ensures availability of lysosomes with minimum damage which otherwise would be caused by prolonged separation procedures and overnight storage. The method will also be useful in preparative investigations since it is standardized to obtain lysosomes from buffalo, beef and lamb kidneys which are commercially available in large quantities.

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Detection of hepatitis B virus in blood samples negative for surface antigen, by DNA probe hybridization assay

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Abstract. A DNA hybridization assay was developed using a cloned hepatitis B viral genome to detect the presence of infectious virions in human serum. The merit of this assay was to put in evidence virus particles in 7 out of 133 sera that were negative for surface antigen (HBsAg) using routine serological methods. The usefulness of this assay was confirmed by actual visualization of the virus under electron microscope. Some serum samples although positive for surface antigen, did not give a hybridization signal by dot blot assay and might indicate cases of acute hepatitis.

Keywords. DNA dot hybridization; hepatitis B virus; integration.

Introduction

Hepatitis B virus (HBV) infection is endemic in many parts of the world (Zuckerman, 1983) and the clinical manifestation associated with HBV infection range from acute hepatitis to more severe chronic stage, eventually leading to hepatocellular carcinoma (Szmuness, 1978; Beasley *et al.*, 1981; Prince, 1983; Hoofnagle *et al.*, 1987; Popper *et al.*, 1987). These studies have indicated relationship between integration of the viral genome into host liver chromosome and development of hepatocellular carcinoma (Koshy *et al.*, 1981; Koch *et al.*, 1984). The integration of the viral DNA is detectable by molecular hybridization technique using cloned HBV genome (Brechot *et al.*, 1980, 1981; Shafritz, 1981; Chen *et al.*, 1982; Ogston *et al.*, 1982; Dejean and Tiollais, 1986). The current diagnosis of HBV infection is based on immunoassays which allow detection of surface antigen (HBsAg), capsid (HBcAg and HBeAg) antigens and their corresponding antibodies. Though these assays are used for the diagnosis and prognosis of the disease and also for the screening of the carriers, they can neither be used for the detection of infectious virions in the serum nor can they differentiate the state of HBV DNA in hepatocytes, that is free or integrated. Cloning of HBV DNA and the development of DNA hybridization assay allow detection of the viral DNA in serum and tissues and provides valuable information for selection as well as for monitoring of patients undergoing treatment (Brechot *et al.*, 1981; Liberman *et al.*, 1983; Scotto *et al.*, 1983; Walter *et al.*, 1987). DNA hybridization assay combined with other immunological assays can distinguish between healthy carriers (not capable of transmitting the infection) and infective chronic carriers (Hoofnagle *et al.*, 1987).

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Abbreviations used: HBV, Hepatitis B virus; RPHA, reverse passive haemaglutination assay; SDS, sodium dodecyl sulphate; SSC, sodium citrate saline.

In the present studies we have analysed some of the factors that influence the process of DNA probe hybridization and the hybridization signal. Evidence is provided for the presence of 42 nm viral particles in blood samples negative for HBsAg by the currently employed serological assays.

Materials and methods

Blood samples

Blood samples for this study were previously screened for the presence of surface antigen (HBsAg) by commercial kits (Hoechst, reverse passive haemaglutination assay, RPHA) and were obtained from Red Cross Blood Bank, New Delhi and G. B. Pant Hospital, New Delhi.

Enzymes and chemicals

Restriction endonucleases used in the studies were purchased from Bethesda Research Laboratory, USA. Protease was obtained from Sigma Chemical Co. St. Louis, Missouri, USA. [^{32}P]dCTP (800 Ci/mM) and nick translation kits were purchased from Amersham, England. Nitrocellulose membrane filters (0·45 μm) were products of Schleicher and Schuell, USA. Other chemicals were of analar grade, obtained from British Drug House, Bombay.

Preparation of radiolabelled probe

Plasmid pCF80 DNA (Lugassy *et al.*, 1987) carrying a tetramer of HBV genome cloned at *Eco*RI site was digested with restriction endonuclease *Eco*RI. A 3·2 kb HBV DNA insert was purified free of pBR322 DNA on low melting agarose gel by electrophoresis (Maniatis *et al.*, 1982) and radiolabelled with [^{32}P]dCTP (Rigby *et al.*, 1977). The specific activity of the probe in different assays ranged from $1-5 \times 10^7$ cpm/μg of DNA.

Isolation of serum DNA

Serum (0·05–1·0 ml) was treated with protease (2 mg/ml) for 4 h in lysis buffer (10 mM Tris, pH 8, 20 mM EDTA, 2% sodium dodecyl sulphate, SDS) and then subjected to phenol-chloroform-isoamyl alcohol extraction. The aqueous phase was separated from phenol phase by low speed centrifugation, from which nucleic acids were precipitated by addition of two volumes of ice-cold ethanol and 1/10th volume of 3 M sodium acetate (pH 5). DNA was pelleted, dried and dissolved in 20 μl of Tris-EDTA buffer (10 mM Tris, pH 8, 1 mM EDTA).

Denaturation of DNA

DNA was denatured by keeping in a boiling water bath for 10 min and cooling rapidly in ice and salt mix.

blot hybridization

ocellulose membrane filters (0·45 µm) were first soaked in 2 × SSC (1 × SSC: M sodium chloride, 0·015 M trisodium citrate, pH 7·2) for 10 min and then air d. Twenty µl of denatured DNA was applied to the membrane by direct spotting Gilson pipetman P20. In the case of dotting under vacuum, denatured DNA in presence of 6 × SSC was adsorbed on the membrane under low vacuum using blot filtration assembly (Bethesda Research Laboratory). DNA was fixed to the brane by baking the filters at 80°C for 2 h. Filters were incubated at 65°C for n 20 ml prehybridization mix (6 × SSC, 0·5 g polyvenyl pyrolidone, 0·5 g bovine n albumin and 0·5 g ficoll 0·1% SDS) to avoid non specific binding of the e. Hybridization was carried out at 65°C for 24 h in the same solution with 0^6 cpm of $[^{32}P]$ labelled 3·2 kb HBV probe. Filters were washed extensively 500 ml of 2 × SSC, 0·1% SDS at 68°C, air dried and exposed to X-ray film)U) at −70°C with the help of intensifying screens (Rege Cine Films). Autographs were developed using Kodak developer and fixer.

hern blot hybridization

from serum separated on 1% agarose gel by electrophoresis was transferred itro-cellulose membrane filters (Southern, 1975). Prehybridization and idization were done as in the case of dot blot hybridization experiment.

ication of HBV particles

ication of the HBV particles was done essentially as described by Petit *et al.*). Thirty ml of plasma positive of HBV DNA by dot blot method, was ifuged at 5,000 *g* for 10 min. The supernatant was taken and HBV particles pelleted by centrifugation for 6 h at 75,000 *g*. The pellet was resuspended in NaCl buffer (50 mM Tris, pH 7·2, 10 mM NaCl), half of the original volume of and recentrifuged 3 times under the same conditions. The final pellet was pended in TN buffer in 1/20th of initial plasma volume and further purified by ifugation at 75,000 *g* over a discontinuous gradient of 10–30% of sucrose ion at 4°C in swing out rotor. The pellet was resuspended in 1/100th of nal volume in TN buffer and centrifuged at 75,000 *g* over 10–60% ntinuous sucrose solution gradient. 0·5 ml fractions were collected and those ive for HBV by dot hybridization were visualized under the electron oscope.

ron microscopy

s particles were identified by transmission electron microscope using negative ing method. Copper grids coated with Formvar and carbon were inverted over s of test serum samples, placed on parafilm for 3 min. Grids were air dried and ined under Philips CM-10 electron microscope at an accelerating voltage of KV.

Results

Factors affecting the sensitivity of molecular hybridization

DNA can be deposited on the nitrocellulose membrane in many ways. A frequently employed procedure is the filteration under vacuum using a dot blot apparatus. Another way would be direct spotting of the DNA by the use of micro-pipette and air drying. Comparison of the signal obtained with the adsorbed DNA by the two methods was investigated (figure 1). The extent of hybridization signal obtained by direct spotting is considerably more as compared to using dot blot apparatus for the same amount of DNA (figure 1A). This was further confirmed by counting the radioactivity present in the spots. Similar observations were made when the DNA was isolated from the sera positive for HBsAg and assayed by both the methods (figure 1B). The higher sensitivity offered by direct spotting could be due to greater retention of the DNA which is not the case when dotting is done by filteration under vacuum.

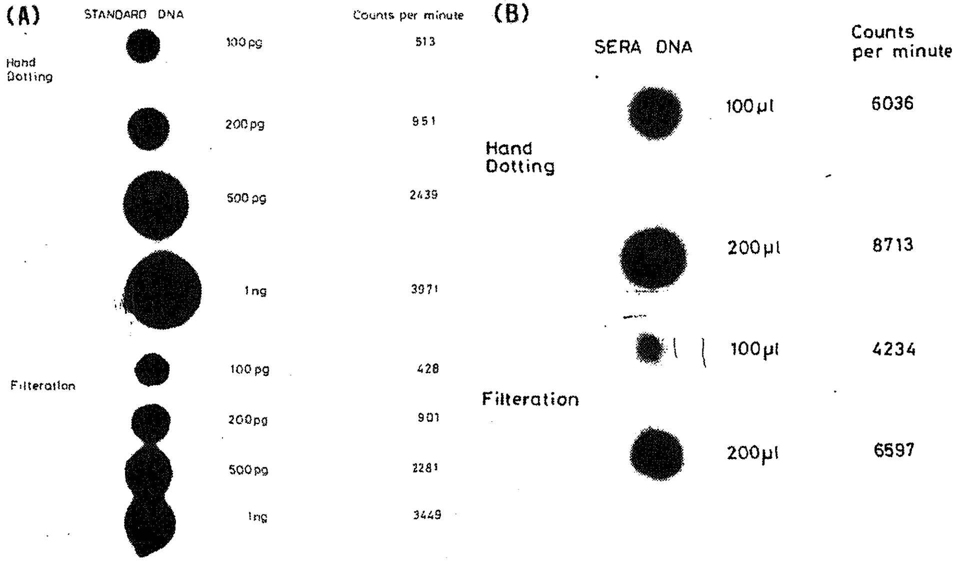

Figure 1. Deposition of HBV DNA by direct spotting or vacuum filteration. (A) 100, 200, 500 pg and 1 ng of 3·2 kb HBV DNA used as probe, was dotted on to nitrocellulose membrane. In one case it was deposited directly as a dot by Gilson pipetman, and in other case it was filtered under vacuum using dot blot apparatus. Filters were hybridized under identical conditions. Figure shows the autoradiographic signal obtained in the two conditions after exposure for 24 h. The radioactivity counts in the spots are given on the right hand side. (B) Same experiment carried out with DNA extracted from 100 and 200 µl from serum of a HBsAg positive patient. Conditions of dotting and hybridization are same as described above.

In order to establish relationship between exposure time and specific activity of the probe, DNA was dotted on to nitrocellulose membrane filter and then hybridized with the probe DNA. The membrane filter was exposed for varying hs of time. Results given in figure 2, show that within 24 h of exposure, it was ible to detect as low as 2 pg of DNA, which is equivalent to 3×10^5 viral cles.

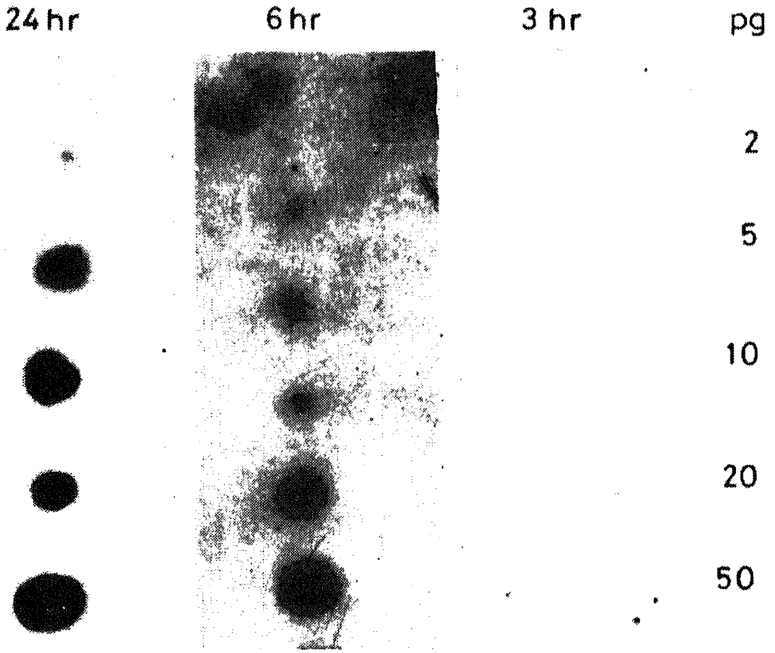

Figure 2. Effect of varying exposure time on the sensitivity of the assay: 2, 5, 10, 20 and 50 pg of 3·2 kb HBV DNA were spotted on to nitrocellulose membrane. Hybridization and washing was done as stated in materials and methods. The filters were exposed for 3, 6 and 24 h.

e authenticity of DNA dot hybridization was further confirmed by a Southern ‹xperiment. DNA from few test sera, that gave positive hybridization signal by lot, were separated on agarose gel, transferred to nitrocellulose membrane and dized with 3·2 kb HBV probe (see methods). For all the sera samples that gave ve hybridization signal by dot blot, a hybridizable fragment of 3·2 kb in size letected (figure 3).

nce of HBV DNA in HBsAg negative sera

der to validate the diagnostic potential of the cloned DNA probe, blood les obtained from voluntary blood donors as well as patients suffering from al hepatitis whose HBsAg status was previously determined by RPHA, were ined by DNA dot hybridization test (figure 4). Seven out of 133 blood samples voluntary blood donors although were RPHA negative for surface antigen ıted the presence of HBV by DNA dot hybridization test.

ce RPHA is a less sensitive test with the detection limit between 20–30 ng of ıg per ml of serum, a more sensitive test (Abbot ELISA, sensitivity 0·5–1 ng)

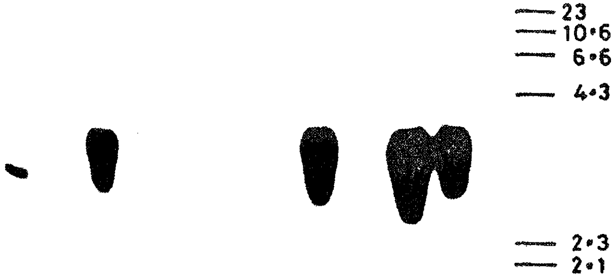

Figure 3. Southern blot hybridization of serum DNA. DNA isolated from 4 positive an 4 negative serum samples as determined by DNA dot blot assay, were analysed b electrophoresis on 1% agarose gel, denatured, neutralized and transferred to nitrocellulos membrane by Southern blot technique (see materials and methods). The filter wa hybridized with purified 3·2 kb HBV DNA fragment and exposed for 24 h. Lanes 3, 7, 9, 1 contain DNA isolated from sera positive by dot blot assay, while lanes 4, 5, 6, 8 had DN isolated from sera negative for HBV by dot blot assay. Lane 1 had probe DNA while lan 2 was the negative control. Position of DNA fragments of *Hind*III digested DNA indicated on the right.

was performed on these samples which also showed the absence of any detectabl HBsAg. DNA dot hybridization results obtained on these samples are presented i figure 4A. Another such case from a different source is shown in figure 4D, case 1

DNA probe analysis of serologically positive cases

Serum samples positive of HBsAg were also examined by dot blot hybridizatio assay. Results are presented in figure 4B, D. Amongst samples suffering fro clinical hepatitis with positive HBsAg status, one did not give hybridization sign (figure 4B, 39).

This was also found to be true with voluntary donor blood samples (figure 4D where out of 8 samples positive for surface antigen, 7 were found to be positive b DNA dot hybridization test. There was, however, one serum which did not gi hybridization signal even at higher serum concentration (figure 4D, case 2).

Electron microscopic studies

The DNA spot test results on HBsAg negative blood samples were furthe confirmed by actual visualization of the virus particles under electron microscop Figure 5A is an electron micrograph of a viral preparation obtained from 30 ml serum sample positive for HBsAg as well as HBV DNA, while figure 5C is a electron micrograph of viral preparation from a case negative for HBsAg by Abb ELISA but positive by DNA dot hybridization test.

Figure 4. Spot blot hybridization on test sera. DNA isolated from serum samples (materials and methods) was spotted on the nitrocellulose membrane and hybridized with 5×10^6 cpm of probe. (A) Autoradiograph of signal obtained with voluntary blood donors whose HBsAg status was negative. (B) Hybridization profile of healthy carriers and patients suffering from clinical hepatitis cases 37, 39, 40, 41, 48, 50, 56 represent clinical hepatitis cases. (C) Hybridization profile of positive and negative control serum. (D) Effect of varying serum amount on the intensity of hybridization on samples obtained from voluntary blood donors from another source. HBsAg status is shown at the top.

sion

ommunication reports the standardization of the DNA probe test which put dence the presence of hepatitis B virions. The test can be performed on um of 0·05 ml of serum with sensitivity of 2 pg of DNA equivalent to 3×10^5 particles and compares favourably with that performed by other investigators ot *et al*., 1982; Scotto *et al*., 1983).

logical assays are invariably employed in blood banks for detection of g, the surface antigen and not the virions detectable by DNA hybridization Normally these two types of assays should give similar conclusions and this dicated in figure 4B, cases 37, 40, 41, 48, 50, 56 and figure 4D, cases 3, 4, 6, 7, 0 (spot 9 not visible in print).

absence of detectable hybridization signal in HBsAg positive sera might te clearance of the virus at the time sample was withdrawn and may indicate of acute hepatitis (figure 4B, cases 39; 4D, case 2). Alternatively, in cases of

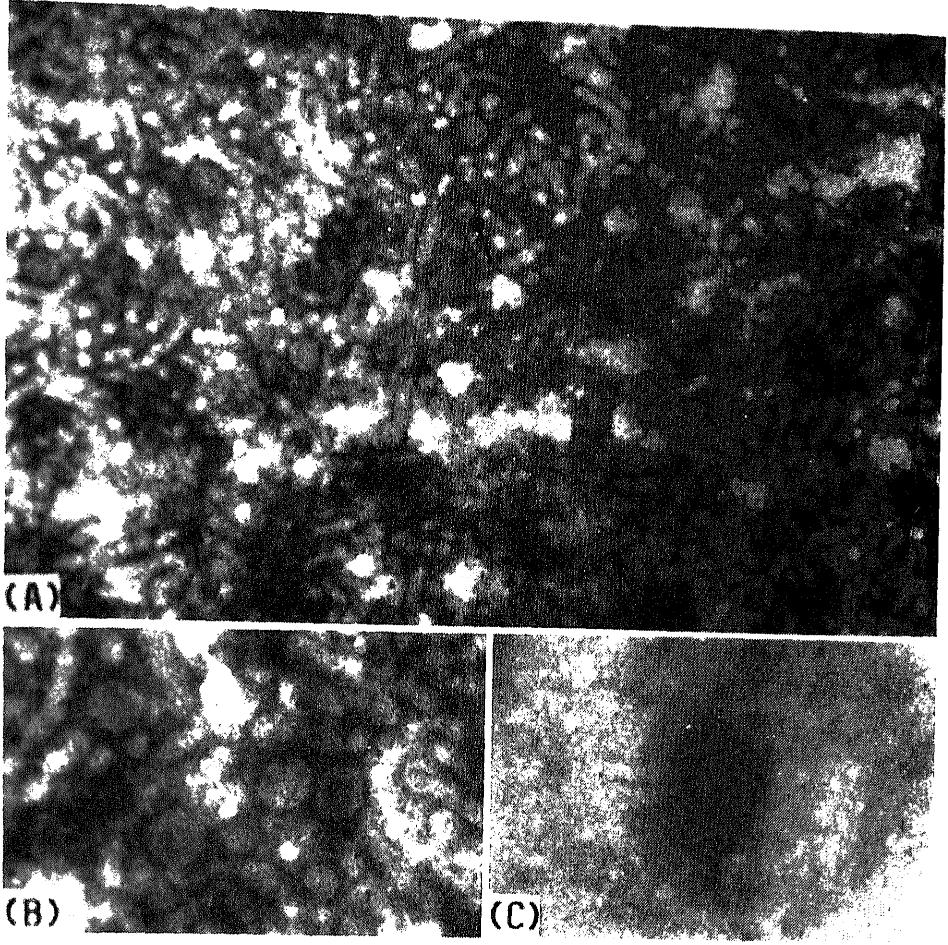

Figure 5. Electron microscopic studies of purified virus preparations. (A) Electron micrographs of purified virus preparation from a serum sample positive for both HBsAg and HBV × 120,000. (B) Enlarged portion of 5A × 200,000. (C) Electron micrograph of purified virus preparation from serum negative for HBsAg but positive for HBV DNA × 200,000.

(^), 42 nm dane particles; (↑), 22 nm spherical HBsAg particles; (⇑), Filamentous HBsAg particles

1984). Hoofnagle *et al.* (1987) Popper *et al.* (1987) have reported the production and release of 22 nm surface particles in the serum without replication of the virus. Such observations may indicate integration of HBV genome into liver chromosome.

Amongst 133 HBsAg negative serum samples analysed by the DNA probe assay, [illegible] gave positive hybridization signal, suggesting the greater sensitivity offered by the assay. Furthermore, this assay allowed the detection of infectious virions in the serum which is not the case with ELISA developed for detection of surface protein. The assay can be useful in detection of infectious pregnant mothers (Okada *et al.*, 1976, Beasley *et al.*, 1977, 1983) and also to study the prevalence of HBV varients.

These variants do not express the same antigen coded by HBV and also differ in e serological pattern. However they show sequence homology at the DNA level. iese variants are presently catagorized under post transfusion related NANB patitis. Furthermore it has been observed that these variants are capable of ısing disease in chimpanzees which is the only experimental model for replication d subsequent manifestation of the disease (Wands *et al.*, 1986; Thiers *et al.*, 1988).

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vivo biocompatibility of aliphatic segmented polyurethane in rabbit

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Abstract. An aliphatic segmented polyurethane with soft to hard segment ratio 3 was synthesised using hexamethylene diisocyanate, polypropylene glycol 400 and 1,4-butane diol. A stainless steel cage implant system has been used to study the *in vivo* biocompatibility of this polyurethane. United States Pharmacopoeia negative control polyethylene was used for the comparison. Three cages, one with polyurethane another with United States Pharmacopoeia polyethylene and the third control empty cage were implanted subcutaneously in the dorsal aspect of rabbits. The inflammatory exudate surrounding the material was aspirated from the cages on 4, 7, 14 and 21 days after implantation. The total protein content in the exudate aspirated from all the 3 cages was significantly higher at 7 days than in the reported normal rabbit serum of New Zealand white rabbit but equal to that of our rabbit colony. The albumin concentration was lower in the initial period but increased at 21 days post implantation period in all the cages. Concentration of α_1, α_2 and γ-globulin also decreased in all cages at 21 days. Neutrophils were predominant in all the exudates aspirated from polyurethane, polyethylene and empty control cages during whole implantation period. This is attributed to the profound effect of the cages on the surrounding vasculature. Macrophage was found to be seen during acute phase of inflammation due to the migration of macrophage along with neutrophil towards the inflammatory lesion. The percentage of neutrophils showed a faster decline in the cage containing polyethylene at 21 days. The extra cellular alkaline phosphatase activity, though higher in exudate from cages containing polyurethane at 14 days post implantation, was same in all 3 cages at 21 days. Leucine amino peptidase activity was found to be decreased at 21 days of post implantation time though the empty control cage exhibited an increase at 14 days post implantation. The inflammatory response at 21 days was similar in polyurethane and the control polyethylene.

Keywords. Cage implants; aliphatic segmented polyurethane; inflammatory exudate; proteins; leukocytes; enzymes; biocompatibility.

oduction

urethane has been widely used for biomedical applications which result in
t term or long term contact between the material and tissues of the body. The
e compatibility of the polyurethane depends upon the response of the cells and
enzymes on the material and *vice versa*. One of the most important factors
h affect the material *in vivo* biocompatibility is the cellular interactions which
r at the material-tissue interface. Histopathological techniques have been
ly adopted to determine the tissue tolerance of material with subcutaneous
ants and intramuscular implants. These histopathological techniques offer the

interactions at the material-tissue interface. It is difficult to investigate material/cell interactions at the interface during different periods of implantation under *in vivo* conditions with the histopathological techniques. Imai *et al.* (1979) have introduced a cell culture biocompatibility test system to study the material and cell interaction. However, this *in vitro* technique does not simulate the dynamic nature of the cellular interaction at the interface. Marchant *et al.* (1983) have adopted a new technique 'cage implant system' in which the candidate material is allowed to bathe in the inflammatory exudate under *in vivo* conditions. The technique enables to assess the level of inflammatory response to an implanted material. The latest report by Spilezewski *et al.* (1988) deals with *in vivo* biocompatibility of catheter materials investigated by the cage system. In all these studies the rat has been selected as the animal model. In our present studies we have selected rabbit as the animal model for *in vivo* biocompatibility of the candidate aliphatic segmented polyurethane using cage implant system.

Experimental

Preparation and characterization of polyurethane

The candidate polyurethane is polyether based. Hexamethylene diisocyanate (HDI) and polypropylene glycol (PPG, molecular weight 400), were used (Fluka A.G.). 1,4-Butane diol (BD) (Merck Schuthardt) was used as the chain extender. Dibutyltin dilaurate (0·05 pbw) from Fluka AQ was used as catalyst. Adequate quantity of dimethyl acetamide was used for transferring the reactants to the reaction vessel. All the reactants were degassed for 30 min before the reactions. Initially a prepolymer was prepared under nitrogen atmosphere using HDI and PPG 400. The reaction was conducted for 30 min under exothermic conditions leading to the maximum temperature of 120–130°C. The reaction mixture was cooled to 30°C. Then the prepolymer was chain extended with BD and allowed to react exothermically leading to the maximum temperature of 110–120°C for 90 min. The polymer liquid was poured in a preheated (60°C) glass plate and cured at 60°C for 12 h, 80°C for 3 h and 100°C for 2 h. The cured polymer was immersed in distilled water at 25°C for overnight. Then the polymer was subjected to soxhlet extraction using carbon tetrachloride, methanol and diethyl ether for removing unreacted components, catalysts etc. The purified polymer was dissolved with dimethyl acetamide (HPLC grade) and cast into film on a clean silicone oil-coated glass plate and dried in air oven at 60–70°C. The casted film was subjected to physicochemical and mechanical testing.

The molecular weight of the polymer was determined by gel permeation chromatography (Waters Associates, USA) using differential refractometer. Vacuum distilled dimethyl acetamide with 0·05% lithium bromide was used as solvent. μ-Styragel columns with pore size 10^5, 10^4 and 10^3Å were used. Differential thermal analyses were carried out using Dupont 990 thermal analyser. The samples were heated from ambient to 400°C in air at the rate of 10°C/min. Tensile tests were carried out using Instron Universal testing machine as per the ASTM standard D 882. The formulation and properties of this polyurethane are given in table 1.

Table 1. Formulation and properties of candidate polyurethane.

I. Formulation	
(i) Hexamethylene diisocyanate	0·25 mol
(ii) Polypropylene glycol (400 molecular weight)	0·15 mol
(iii) 1,4-Butane diol	0·05 mol
II. Properties	
(i) Molecular weight $\bar{M}n$	270338 ± 20,000
$\bar{M}w$	350600 ± 30,000
(ii) Tensile strength (kg/cm^2)	45 ± 2
Elongation (%)	466 ± 50
(iii) Softening range (°C)	145 − 325
Recrystallization temperature (°C)	66·5

eparation and implantation of polyurethane cages

e stainless steel cages were fabricated using stainless steel wire mesh (austenitic inless steel supplied by M/s. Shieves India, Bombay. The stainless steel type 316 ntaining 71·9% α-Fe, 18% Cr, 8% Ni, 2% Mo and 0·1% C. The mesh size was 24 d the wire diameter was 0·015 in. The cage length was 3·5 cm and diameter was m. The cage was fabricated into cylindrical shape. All wire ends were directed o the cage and then compressed along the inner surface seams. The finished cages re cleaned with benzene in soxhlet extractor and then with soap water and ionized water ultrasonically. The nonporous and smooth candidate polyurethane, s cleaned ultrasonically in ether and cut into rectangular specimens of length m, width 0·5 cm and thickness 0·5 mm. The material was put into the cage and aned once again. These cages were packed in clean room and sterilized with γ-diation with 2·5 M rad dose. The cage with polyurethane was code named as K_3 lyurethane. A second set of cage was prepared for biomedical grade polyethylene pplied by United States Pharmacopoeia (USP) Inc., Rockville, Maryland, USA, 5) as negative control. The cage with this polyethylene was code named as K_4 lyethylene. A third set of empty cage was also prepared to serve as control to th K_3 polyurethane and K_4 polyethylene. This was code named as K_7 empty ge. Albino rabbits from the Institute colony were used. They were maintained on nd Lever Pellets and water *ad libitum.* The animals were anaesthetized and epared for surgery. Two cages, one with candidate polyurethane (K_3) and another th USP negative control polyethylene (K_4) and a third empty cage (K_7) control ere selected for implantation subcutaneously in the dorsal aspect of each animal. single incision, 2 cm in length was made on the midline. Blunt scissor dissection as then used to create implant sites by tunneling subcutaneously in the lateral sition of cephaled and caudal direction. The cage implant K_3 was then inserted, e end of the cage being 2·5 cm from the incision and the incision was sutured. milarly K_4 and K_7 cages were implanted through two other subcutaneous ckets. Three animals were used for these studies.

nalysis of exudate

ne pale yellow inflammatory exudate that accumulated within the cages were riodically aspirated at the post implantation period of 4, 7, 14 and 21 days under sterile conditions using a 22 gauge needle with a syringe. The exudate collected each time was not more than 0·5 ml. All samples were tested for sterility. Two drops of exudate collected asceptically were inoculated into 10 ml of fluid thioglycollate medium (Hi-media Laboratories). The inoculated medium was incubated at 35°C. This was observed daily for 7 days for bacterial growth, turbidity and change of pH of the media. If the growth and turbidity increased with time, exudate was declared as contaminated. The exudate was analysed for total leucocyte count, differential cell count, lysosomal enzymes and total protein. Quantitative leucocyte count was carried out using a haemocytometer and differential leucocyte count was accomplished by staining the smears with Leishman stain. Alkaline phosphatase was measured using disodium phenyl phosphate as substrate as per the method described elsewhere (Varley, 1967). Total acid phosphatase, prostatic acid phosphatase and leucine amino peptidase were determined using Sigma kit 104 and 251 respectively. Total protein content was determined by the biuret method (Reinhold, 1953). Protein distribution was estimated by cellulose acetate electrophoresis using Cosmos (Japan) electrophoretic apparatus. At the end of 21 days of implantation time the animals were sacrificed and the cages were removed, opened, and implanted cage and surrounding tissues were examined macroscopically (figure 1).

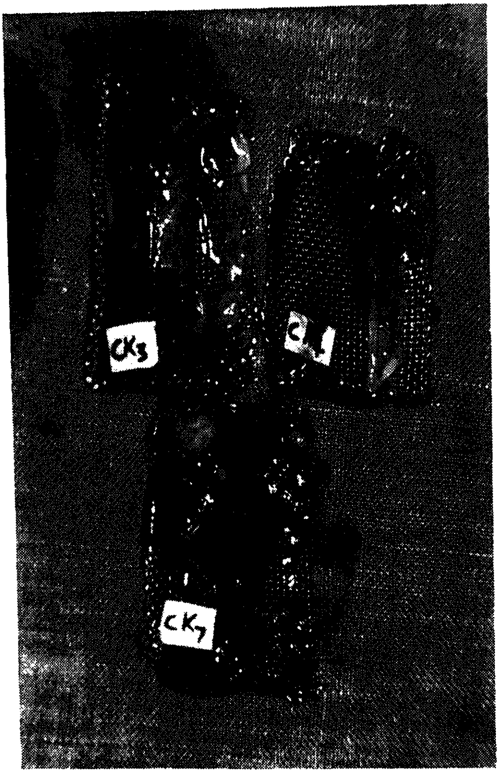

Figure 1. K_3, K_4 and K_7 cages with polymer strip harvested from rabbit.

Results

more general way governs the total response of blood and tissue to the polymer implants. The results of the protein analysis of the exudate are given in table 2. At 4 days post implantation time, it was not possible to aspirate the exudate except in one of the K_7 empty cages.

The extracellular total protein content in each exudate was determined and no significant differences were observed in K_3 polyurethane, K_4 polyethylene and K_7 empty cages at 7 days. After 21 days implantation period, the total protein content decreased. The total protein content at 7 days was significantly higher in the exudate than in the normal rabbit serum (5·2 ± 0·79 g/dl) according to the values obtained by Kozma *et al.* (1967). However, the total protein content was equal to that in normal rabbit serum of our rabbit colony (9 ± 0·5 g/dl). The electrophoretic distribution of the protein is also given in table 2. At 7 days the percentage concentration of α_2, β and γ-globulin in the exudate were higher than normal rabbit serum values of New Zealand rabbit and our rabbit colony while the value for albumin was lower. However the values for α_1, α_2, β and γ decreased appreciably from the original value while the value for albumin increased at 21 days. At this period the K_3 polyurethane, K_4 polyethylene and K_7 empty cage have more or less same albumin value.

The results on the white cell analysis of the exudate are given in table 3. The differential cell counts are given in tables 4 and 5. The collection of exudate was carried out at around 10 AM in the collection day to avoid the diurnal variation in differential cell counts (Kozma *et al.*, 1974). Neutrophil is the predominant cell in the exudate at all periods in K_3 polyurethane, K_4 polyethylene and K_7 empty cages. However the absolute value does decrease appreciably at the completion of 21 days of implantation. Macrophages along with neutrophils were seen in one of the exudates of the empty cages at the end of the period, 4 days. This is attributed to the fact that macrophage initially migrate into the bacterial inflammatory sites simultaneously with neutrophil in rabbit (Issekutz *et al.*, 1981). The percentage of macrophage started increasing in the latter period even in K_4 polyethylene and K_7 empty cages. This is due to the continued migration of macrophage into the inflammatory lesion long after the neutrophil have stopped (Issekutz *et al.*, 1981). The increase in the macrophage concentration in K_3 polyurethane cages from 7 to 14 days indicates the resolution of acute inflammatory response and start mild chronic response.

While the cellular interactions appeared to be different for all K_3, K_4 and K_7 cages, significant differences were also observed in the extra cellular alkaline phosphatase activity (figure 2). The results indicate increased activity for K_3 polyurethane at 14 days of post implantation time while the values for K_4 polyethylene and K_7 empty cages decreased. Since the alkaline phosphatase is localized to the specific granules of polymorphonuclear leucocytes, activity of this enzyme is an important parameter to judge acute phase of inflammation. The activities of total acid phosphatase and 'prostatic' acid phosphatase reflect both the acute and chronic phase of inflammation. The total acid phosphatase activity decreased in all cages with implantation period (table 6). While the prostatic acid phosphatase activity increased for K_3 polyurethane and K_4 polyethylene cages at 21 days, significant differences were observed between K_3 and K_4 cages in the initial period of inflam-

Table 2. Electrophoretic distribution (%) of total proteins in the exudate with implantation time.

Implantation time (days)	Total protein g/dl			Albumin (%)			α_1 (%)			α_2 (%)			β (%)			γ (%)		
	K_3	K_4	K_7	K_3	K_4	K_7	K_3	K_4	K_7	K_3	K_4	K_7	K_3	K_4	K_7	K_3	K_4	K_7
4	—	—	7·6 (1)	—	—	28·9 (1)	—	—	14·1 (1)	—	—	7·4 (1)	—	—	9·1 (1)	—	—	40·5 (1)
7	8·60 (1)	8·45 ± 0·75 (2)	8·17 ± 1·47 (3)	31·3 (1)	40·2 ± 8·2 (2)	37·6 ± 1·1 (3)	12·6 (1)	10·3 ± 3·8 (3)	11·5 ± 0·7 (3)	10·7 (1)	14·75 ± 5·15 (3)	8·9 ± 1·1 (3)	12·4 (1)	8·3 ± 1·9 (3)	9·9 ± 4·0 (3)	33 (1)	31·9 ± 2·8 (3)	32·1 ± 6·9 (3)
14	—	7·6 (1)	6·70 (1)	—	50 (1)	54·9 (1)	—	13·2 (1)	8·5 (1)	—	8·8 (1)	9·9 (1)	—	8·7 (1)	5·7 (1)	—	19·3 (1)	21·0 (1)
21	6 ± 1·1 (3)	5·5 (1)	7·1 (1)	61·4 ± 3·8 (3)	68·7 (1)	54·5 (1)	6·9 ± 1·6 (3)	6·4 (1)	5·4 (1)	7·0 ± 2·2 (3)	5·6 (1)	7·6 (1)	16·7 ± 3·1 (3)	7·1 (1)	9·0 (1)	14·0 ± 5·7 (3)	22·2 (1)	23·5 (1)
New Zeland white rabbit serum (Kozma *et al.*, 1967)		5·2 ± 0·79			56·94			8·90						9·94			11·87	
Institute rabbit serum		9 ± 0·5 (2)			64·9 ± 0·3 (2)			16·9 ± 0 (2)			3·75 ± 0·25 (2)			7·3 ± 0·5 (2)			7·15 ± 0·45 (2)	

Numbers in parentheses indicate number of samples.

Table 3. Variation in total leucocyte concentration in the exudate with implantation time.

Implantation time (days)	Leucocyte concentration (cells/mm^3)		
	K_3	K_4	K_7
4	—	—	1150 (1)
7	1250 (1)	2725 ± 425 (2)	2517 ± 633 (3)
14	1350 (1)	800 ± 150 (2)	450 (1)
21	1517 ± 1004 (3)	633 ± 247 (3)	550 (1)

4. Variation in neutrophil and eosinophil concentration in the exudate with implantation time.

ntation time	K_3		K_4		K_7	
	Neutrophil	Eosinophil	Neutrophil	Eosinophil	Neutrophil	Eosinophil
	—	—	—	—	1104 (1)	0 (1)
	1107 ± 75 (2)	25 ± 6 (2)	2384 ± 123·0 (2)	0 (2)	2013 ± 428 (3)	50 ± 25 (3)
	999 ± 26 (2)	0 (2)	600 (1)	0 (1)	450 (1)	0 (1)
	1062 ± 318 (3)	0 (3)	519 ± 76 (3)	0 (3)	511 (1)	0 (1)

cells/mm^3

5. Variation in macrophage and lymphocyte concentration in the exudate with implantation

ıntation time)	K_3		K_4		K_7	
	Macrophage	Lymphocyte	Macrophage	Lymphocyte	Macrophage	Lymphocyte
	—	—	—	—	46 (1)	0 (1)
	37 ± 25 (2)	81 ± 43 (2)	259 ± 95 (2)	82 ± 27 (2)	152 ± 126 (3)	302 ± 264 (3)
	290 ± 7 (2)	61 ± 7 (2)	136 (1)	64 (1)	0 (1)	0 (1)
	227 ± 226 (3)	228 ± 0 (3)	95 ± 57 (3)	19 ± 18 (3)	39 (1)	0 (1)

: cells/mm^3

ion as observed from the values of leucocyte. Leucine amino peptidase activity decreased with implantation time for K_3 polyurethane and K_4 polyethylene es in comparison with that of K_7 empty cage at 14 days of implantation (figure 3).

cussion

cage implant system offers an understanding of the acute and chronic phase of

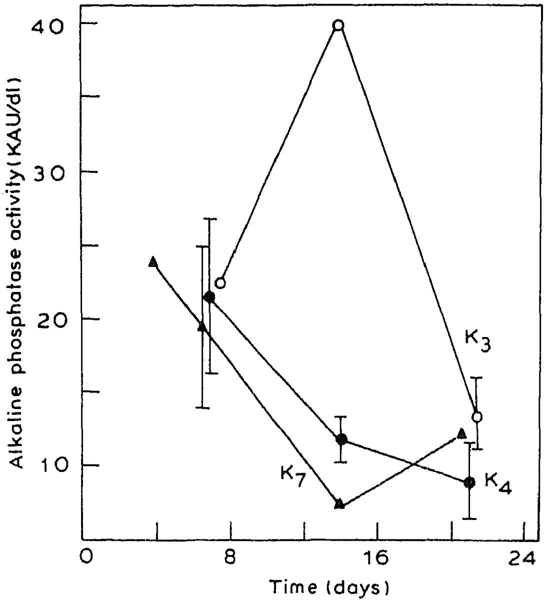

Figure 2. Variation of extracellular alkaline phosphatase activity in the exudate wit implantation time.

Table 6. Variation in extracellular total acid phosphatase and prostatic acid phosphatase activity o the exudate with implantation time.

Implantation time (days)	Total acid phosphatase activity, (SU/ml)			Prostatic acid phosphatase activity, (SU/ml)		
	K_3	K_4	K_7	K_3	K_4	K_7
7	2·2 (1)	—	1·9 ± 0·54 (3)	0·01 (1)	—	0·11 ± 0·1 (3)
14	—	1·13 ± 0·19 (2)	0·49 (1)	—	0·135 ± 0·08 (2)	0·18 (1)
21	1·7 ± 0·7 (2)	1·40 ± 0·8 (2)	—	0·53 ± 0·34 (3)	0·405 ± 0·04 (2)	—

inflammation which gives insight to the material as well as tissue response. Th changes in the exudate during the implantation can be understood with the variatio in total protein, protein distribution, cells and enzymes concentration. During th initial phase of inflammation albumin concentration decreased and α_1- and α_2 globulin concentration increased in comparison with the reported values of Nev Zealand white rabbit serum. The decrease in α_1, α_2 and γ-globulin concentratio and increase in albumin concentration in the latter period of implantation indicat the resolution of acute phase of inflammation (Marchant *et al.*, 1983).

The level of inflammatory reaction to an implant is used to assess the biocompat bility of the implant. The acute phase of inflammatory reaction induced by th polymer implant in the cage is characterised by the predominance of polymorpho nuclear leucocytes. The chronic phase of inflammation is characterized by th predominance of mononuclear leucocytes including macrophages and lymphocyte Predominance of neutrophil in all K_3 polyurethane, K_4 polyethylene and K_7 empt

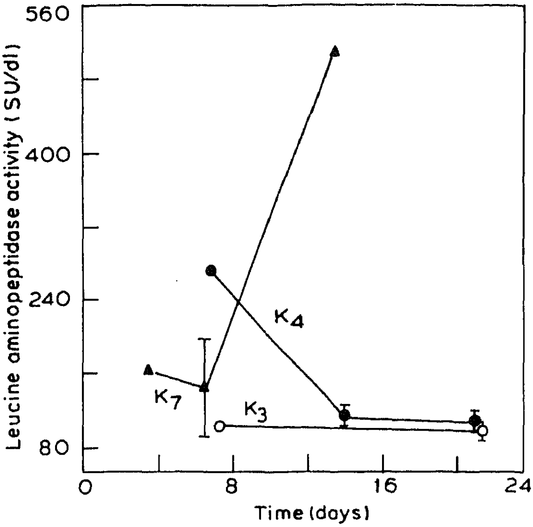

Figure 3. Variation in extracellular leucine aminopeptidase activity in the exudate with implantation time.

during the whole implantation time is a noted feature. It has been observed e neutrophils stop migrating into the bacterial lesions after 8 h in rabbits tz and Movat, 1980). Moreover the neutrophils in tissues have shorter life e at the site of inflammation (Issekutz *et al.*, 1980). Therefore the continued ninance of neutrophil in all the K_3, K_4 and K_7 cages is due to the profound y the stainless steel cages on the surrounding vasculature as observed in the pure metals by McNamara and Williams (1981). The K_3 polyurethane cage s lower concentration of leucocytes in 7 days. The K_4 polyethylene and K_7 cages exhibit lower concentration of leucocytes in the latter period of implan- indicating the faster resolution of inflammatory response. The leucocyte value ed more or less same for K_3 polyurethane cage during the implantation .

ntly importance has been given to growth factors and inhibitors produced nonuclear phagocytes and their role in the chronic inflammatory reaction. growth factors produced by monocytes and macrophages and other inflam- cells have implications in the implant associated wound healing responses n, 1987). These growth factors could be released upon activation of the phage and induce the proliferation of fibroblasts around the cage implant to capsule.

presence of extra cellular acid phosphatase and Leucine amino peptidase es the cellular activation during the chronic phase of inflammation.

his study the alkaline phosphatase activity at day 7 is more or less same for lyurethane, K_4 polyethylene and K_7 empty cages. Though K_4 and K_7 have number of neutrophils at day 7 compared to K_3, all the cells were not ed. This may be due to the migration of cells to the wound site in response motactic stimuli as reported by Spilezewski *et al.* (1988). By day 14, K_3 ethane has higher value for extracellular alkaline phosphatase activity in

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Hypolipidemic principle of the inflorescence stalk of plantain (*Musa sapientum*)

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Abstract. A pectin present in the juice of the inflorescence stalk of plantain (*Musa sapientum*) has been isolated. The material contained 32·4% hexoses and 52·5% uronic acid. On administration to rats fed both cholesterol free and cholesterol diet, this material showed significant lowering of cholesterol and triglycerides in the serum, liver and aorta. There was decreased cholesterogenesis in the liver as was evident from decreased activity of hydroxymethylglutaryl coenzyme A reductase and decreased incorporation of labelled acetate into hepatic cholesterol. Hepatic bile acids showed significant increase and there was increased fecal excretion of neutral sterols and bile acids. Release of lipoproteins into the circulation was lower. The material also caused increase in the activity of lipoprotein lipase in the heart and adipose tissue and also of plasma lecithin:cholesterol acyl transferase.

Keywords. *Musa sapientum*; cholesterol; triglycerides; HMG CoA reductase; lipoprotein lipase; plasma LCAT.

Introduction

The stem (inflorescence stalk) of plantain (*Musa sapientum*) is widely used as a vegetable in south India. The juice of the stem is claimed to have beneficial effects in reducing obesity by local practitioners of naturopathy. This fact led us to study the effect of the juice of the stem on the level of lipids (cholesterol and triglycerides) in the serum and tissues in rats fed cholesterol free and atherogenic diet. It was found in these preliminary experiments that administration of the juice resulted in significant lowering of cholesterol and triglycerides in the serum and tissues in these rats. This paper describes the results of studies on the isolation of the hypolipidemic principle from the juice and the mechanism of this effect.

Materials and methods

Preliminary studies showed that a polysaccharide containing uronic acid was responsible for at least some of the activity exhibited by the juice. The following procedure was therefore adopted for its isolation.

Fresh juice of the pith of the stem of plantain was treated with cetyl pyridinium chloride (CPC) to a final concentration 1% CPC. CPC formed an insoluble complex with the polysaccharide. The complex was collected by centrifugation, dissolved in 3 M $MgCl_2$ solution and the polysaccharide precipitated by the addition of 4–5 volumes of ethanol. Since this material contained small amounts of protein, it was digested with papain (crystalline papain, 1/3rd the dry weight of the material) in 0·1 M phosphate buffer pH 6·5, containing 0·005 M EDTA and 0·005 M cysteine for 48 h at 65°C, with change of papain every 16 h. The papain digest was deproteinised with trichloroacetic acid (TCA) (final concentration 10%) and the supernatant dialysed till free of TCA. The polysaccharide was precipitated from the solution by 4–5 volumes of 95% ethanol containing 1–2% potassium acetate. The precipitate collected by centrifugation was dried in vacuum. The yield of this material was 1·5 g per litre of the juice.

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Total uronic acid was estimated in the material by the modified procedure of Bitter and Muir (1962). Total hexose was estimated by the phenol-sulphuric acid method (Dubois *et al.*, 1966).

Animal experiments

Composition of cholesterol free diet (g/100 g)

Corn starch	71
Casein (vitamin and fat free)	16
Groundnut oil	8
Salt mixture	4
Vitamin mixture	1

Composition of high fat cholesterol diet (g/100 g)

Corn starch	62
Casein (vitamin and fat free)	16
Coconut oil	15
Salt mixture	4
Vitamin mixture	1
Cholesterol	2

The composition of the salt mixture and vitamin mixture has been described before (Menon and Kurup, 1976).

Rats fed cholesterol free diet: Male albino rats (Sprague–Dawley strain, weighing 80–100 g) receiving cholesterol free diet were divided into groups 1 and 2 of 12 rats each. Group 1 served as control while the group 2 was given the test material (20 mg/100 g/day orally for 45 days).

Rats fed high fat-cholesterol containing diet: In another experiment, two groups of rats (weighing 80–100 g) receiving high fat cholesterol diet were divided into groups 3 and 4 of 12 animals each. Group 3 served as control while group 4 received the test material (20 mg/100 g/day orally for 90 days).

The material dissolved in water (1 ml) was administered daily to the animals of

·ther increase in cholesterol lowering action at higher levels. At the end of the ›perimental period, the rats in each group were starved overnight and killed by ınning and decapitation.

Cholesterol and triglycerides were estimated in the serum and tissues as described rlier (Leelamma *et al.*, 1978). Extraction of liver bile acids was carried out :cording to the procedure of Okishio *et al.* (1967) and bile acids estimated zymatically using 3 α-hydroxysteroid dehydrogenase (Robert, 1969). Changes β-hydroxymethylglutaryl coenzyme A (HMG CoA) reductase activity (EC 1·1·34) tissues were estimated as described by Venugopala Rao and Ramakrishnan 975) by determining the ratio of HMG CoA: Mevalonic acid. Separation of serum ›oproteins was carried out by the procedure of Warnick and Albers (1978) and at of Burstein and Scholnick (1973). Very low density lipoprotein (VLDL) was ecipitated by sodium dodecyl sulphate (final concentration 0·7%) and LDL + low density lipoprotein (LDL) by the heparin and manganese precipitation ethod (final concentration heparin 0·144% and $MnCl_2$ 0·091 M). Release of ›oproteins into the circulation was studied using Triton WR 1339 as described ırlier (Lakshmi Prabha *et al.*, 1988). Fecal neutral sterols and bile acids were :tracted by the general procedure of Grundy *et al.* (1965). Neutral sterols and bile :ids were estimated as mentioned above. Activity of lipoprotein lipase (LPL) of e heart and adipose tissue was determined according to the procedure of Krauss *al.* (1974). Protein was determined by the method of Lowry *et al.* (1951). *In vivo* corporation of 1,2-[^{14}C] acetate into tissue lipids was carried out as described ırlier (Molly Thomas *et al.*, 1983). Assay of plasma lecithin: cholesterol acyl ansferase (LCAT) (EC 2·3·1·3), was carried out as described by Annie Abraham ıd Kurup (1988).

For histochemical examination, the tissues from the rats fed cholesterol free diet ere removed to 10% buffered formalin. Sections were stained with heamatoxylin ıd eosin.

esults and discussion

he material on analysis contained 32·4% total hexoses and 52·5% uronic acid. It ıerefore appears to be a pectin. It is free from protein. Pectins from several sources ave been reported to contain condensed tannins, which are rather difficult to ɜmove (Fry, 1983; Selvendran *et al.*, 1985). Estimation of tannins in the material ccording to the procedure of Cameron *et al.* (1943) showed that it contained 3–4% f tannins.

Diet consumption was similar (10·5 ± 1·5 g) in the rats of groups 1 and 2 and also ı 3 and 4 (11·5 ± 1·3 g). However, gain in weight was significantly lower in the rats ;iven the material (45 ± 1·2 and 25 ± 1·5 g in the control and experimental rats espectively in the cholesterol free diet group and 90 ± 2·5 and 68 ± 2·8 g respectively ı the cholesterol diet group). The liver weight per 100 g body weight was however omparable in the rats of the control and experimental animals both in the :holesterol free and cholesterol diet groups (3·01 ± 0·24 g in the cholesterol free diet abnormality in the experimental groups. No sign of necrosis was evident indicating that the material is non-toxic at the dose used (figures 1 and 2).

Concentration of cholesterol and triglycerides in the serum and tissues

Concentration of cholesterol was significantly lower in the serum, aorta and liver in the rats given the material in both the cholesterol free and cholesterol diet groups (table 1). Triglycerides were also significantly lower in the serum and these tissues and also in the adipose tissue in the rats given the material.

Concentration of cholesterol in the serum lipoproteins

The concentration of cholesterol in VLDL, LDL and high density lipoprotein (HDL) was significantly lower in the rats given the material in both cholesterol free and cholesterol diet groups (table 2). Thus the material shows significant cholesterol and triglyceride lowering action in the serum and tissues. The hypocholesterolemic action in the serum was manifested in all the lipoprotein fractions.

Activity of HMG CoA reductase

The activity of HMG CoA reductase was significantly lower in the liver in the rats given the material in the cholesterol free diet groups (table 2). The incorporation of label into free and ester cholesterol in liver was also significantly lower. This is understandable since the activity of HMG CoA reductase generally correlates very closely with the rate of cholesterol biosynthesis. The material causes decreased cholesterogenesis in the liver of normal animals.

Concentration of bile acids

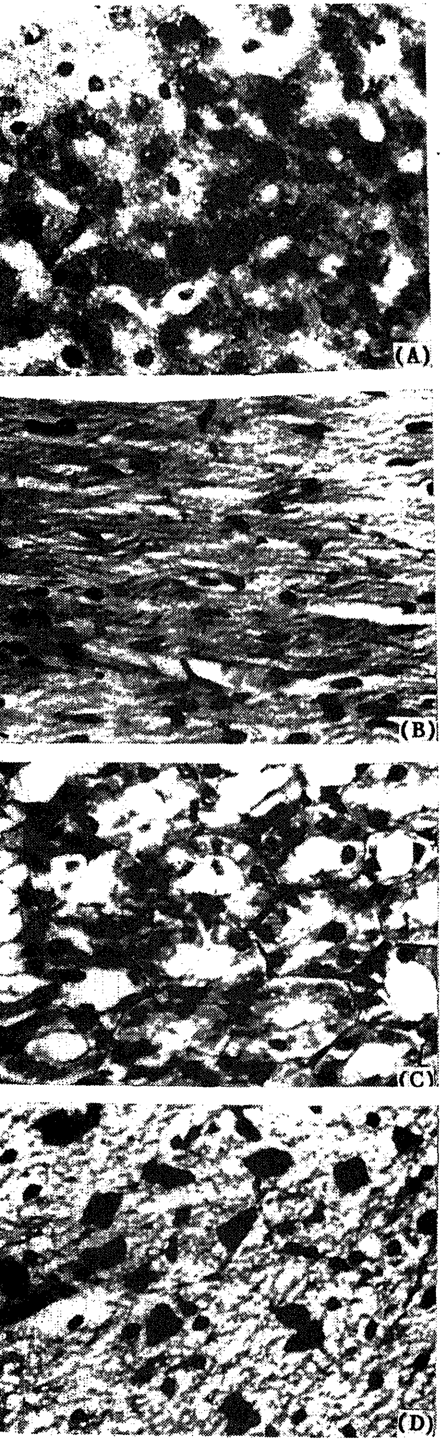
Concentration of total bile acids in the liver showed significant increase in the liver in the rats given the material in both cholesterol free and cholesterol diet groups (table 3). There was also increased fecal excretion of neutral sterols and bile acids in these rats. The increased concentration of hepatic bile acids may probably be due to increased hepatic degradation of cholesterol to bile acids.

Thus the hypocholesterolemic activity of the material in rats fed cholesterol free diet may be due to decreased hepatic cholesterogenesis, increased degradation of cholesterol into bile acids in the liver and increased fecal excretion of neutral sterols and bile acids. In rats fed cholesterol diet also, increased degradation of cholesterol to bile acids and fecal excretion of neutral sterols and bile acids may be responsible for the cholesterol lowering action. Cholesterogenesis in rats fed cholesterol diet was not studied.

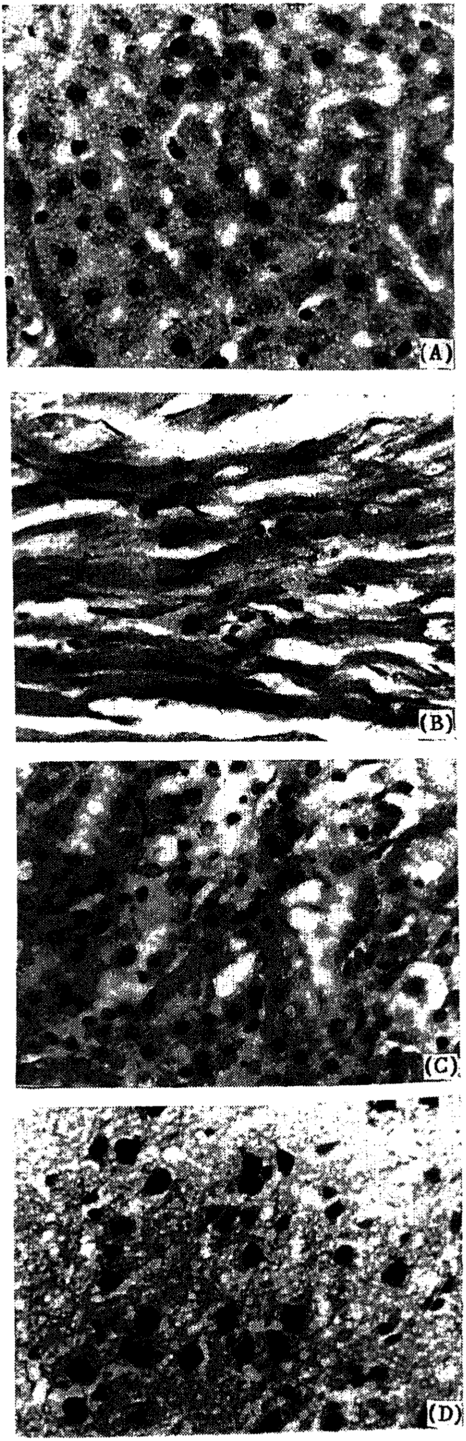
Release of lipoproteins into the circulation

There was decreased release of lipoproteins into the circulation in the rats given the material in both the cholesterol free and cholesterol diet groups (table 4). The lower

(1)



(2)



Figures 1 and 2. Cells from liver (A), heart (B), kidney (C) and brain (D)

1. Concentration of cholesterol and triglycerides in serum and tissues.

	Cholesterol			Triglycerides (expressed as triglyceride glycerol)			
ps	Serum (mg/100 ml)	Liver (mg/100 g wet tissue)	Aorta (mg/100 g wet tissue)	Serum (mg/100 ml)	Liver (mg/100 g wet tissue)	Aorta (mg/100 g wet tissue)	Adipose tissue (mg/100 g wet tissue)
sterol free diet							
ontrol	69·0 ± 2·0	367·0 ± 12·0	187·0 ± 6·0	6·0 ± 0·0	392·0 ± 12·0	647·0 ± 20·0	5154·0 ± 164·0
xperimental	46·15 ± 1·02[a]	270·0 ± 9·45[a]	152·9 ± 4·8[a]	5·1 ± 0·16[a]	257·6 ± 8·24[a]	511·0 ± 22·75[a]	3910·45 ± 125·13[a]
sterol diet							
ontrol	125·3 ± 4·01	1435·2 ± 50·2	387·1 ± 13·55	14·8 ± 0·47	797·9 ± 25·53	1730·3 ± 55·4	7864·52 ± 251·67
xperimental	99·16 ± 3·5[a]	1078·4 ± 37·7[a]	326·0 ± 11·41[a]	10·2 ± 0·33[a]	548·2 ± 17·54[a]	1216·1 ± 38·92[a]	5263·25 ± 168·4[a]

are the mean ± SEM for 12 rats. Group 2 has been compared with group 1 and group 4 with group 3. [a]$P < 0{\cdot}01$.

2. Concentration of cholesterol in the serum lipoproteins, activity of HMG CoA reductase and oration of label into hepatic cholesterol.

	Concentration of cholesterol in serum lipoproteins HDL	LDL (mg/100 ml serum)	VLDL	Activity of hepatic HMG CoA reductase (ratio of HMG CoA to mevalonate*)	Incorporation of 1,2-[^{14}C] acetate into cholesterol in liver Free (counts/min/g tissue)	Ester
sterol free diet						
Control	51·7 ± 1·65	15·2 ± 0·49	7·3 ± 0·24	2·2 ± 0·08	1154 ± 40·39	352 ± 12·32
Experimental	36·1 ± 1·15^a	7·61 ± 0·24^a	3·12 ± 0·09^a	2·8 ± 0·09^a	958 ± 33·53^a	216 ± 7·56^a
sterol diet						
Control	69·41 ± 2·2	41·02 ± 1·31	10·5 ± 0·34	NS	NS	NS
Experimental	57·23 ± 1·8^a	21·67 ± 0·69^a	5·34 ± 0·17^a	NS	NS	NS

ler ratio indicates higher enzyme activity.

s are the mean ± SEM for 12 rats. Group 2 has been compared with group 1 and group 4 with 3. $^a P < 0·01$.

ot studied.

Table 3. Concentration of bile acids in the liver and fecal excretion of neutral sterols and bile acids.

Groups	Bile acids Liver (mg/100 g tissue)	Fecal bile acids (mg/24 h/rat)	Fecal sterols
Cholesterol free diet			
1. Control	31·7 ± 1·11	29·7 ± 1·04	28·6 ± 1·2
2. Experimental	53·3 ± 1·65^a	40·1 ± 1·44^a	39·8 ± 1·8^a
Cholesterol diet			
3. Control	44·6 ± 1·56	38·4 ± 1·34	64·5 ± 2·6
4. Experimental	77·5 ± 2·71^a	66·2 ± 2·32^a	88·9 ± 3·2^a

Values are the mean ± SEM for 12 rats. Group 2 has been compared with group 1 and group 4 with group 3. $^a P < 0·01$.

4. Release of lipoproteins into circulation, activity of LPL and plasma LCAT.

os	Release of lipoproteins into circulation*	Activity of LPL Heart (μmol of glycerol/h/g protein)	Adipose	Activity of plasma LCAT (percentage increase in the ratio of ester cholesterol to free cholesterol during incubation)
sterol free diet				
Control	98·9 ± 3·17	26·6 ± 1·2	112·9 ± 4·6	24·4 ± 0·78
Experimental	71·93 ± 2·31^a	38·9 ± 2·3^a	176·8 ± 7·6^a	28·9 ± 0·93^a
sterol diet				
Control	128·8 ± 4·12	18·8 ± 1·6	88·8 ± 4·3	NS
Experimental	107·0 ± 3·42^a	29·6 ± 1·8^a	132·6 ± 6·4^a	NS

es expressed as percentage increase in cholesterol in the serum in the Triton administered rats over ntrol rats given saline.

s are the mean ± SEM for 12 rats. Group 2 has been compared with group 1 and group 4 with 3. $^a P < 0·01$.

Not studied.

concentration of lipids in the serum may also be due to this decreased release of lipoproteins into the circulation.

Activity of lipoprotein lipase in the heart and adipose tissue and that of plasma LCAT

The activity of LPL in heart and adipose tissue showed significant increase in the rats given the material in both cholesterol free and cholesterol diet groups (table 4). This enzyme is involved in the uptake of circulating triglyceride rich lipoproteins (chylomicrons and VLDL) by the extra hepatic tissues. The increase in the activity of this enzyme may mean increased uptake of these lipoproteins and the decrease in the concentration of serum triglycerides may be due to this.

There was significant increase in the activity of plasma LCAT in the rats administered the material in the cholesterol free diet group. This enzyme is believed to be involved in the transport of cholesterol from the tissues to the liver for its catabolism and the decrease in the concentration of cholesterol in the tissues may agree with the increased activity of this enzyme in rats fed cholesterol free diet. The activity of this enzyme in rats given cholesterol diet was not studied.

There are a number of reports that pectin from various sources show hypocholesterolemic action (Bobek and Chrovathova, 1984; Frank *et al.*, 1986; Judd *et al.*, 1985; Kelly *et al.*, 1978). Most of these pectins have been reported to show activity only at a much higher level (Frank *et al.*, 1986) (500 mg and above/100 g body weight). The material now obtained from the juice of the stem of plantain, on the other hand, shows very significant lipid lowering effect at 20 mg/100 g body weight level. However this material may not be the only active substance present in the juice of plantain. This is because the juice has been found to be effective in clinical trials at a dose of about 500 ml/day. Either the juice may contain some other active principle also or it may reflect species difference, rat (a species normally resistant to atherosclerosis) requiring a higher dose.

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roved solid phase synthesis of luteinizing hormone releasing mone analogues using 9-fluorenylmethyloxycarbonyl amino acid ve esters and catalytic transfer hydrogenation with minimal side- in protection and their biological activities

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Abstract. Using mainly 9-fluorenylmethyloxycarbonyl amino acid 2,4,5-trichlorophenyl esters in the presence of 1-hydroxybenzotriazole and the solid support *p*-alkoxybenzyl alcohol resin, synthesis of luteinizing hormone releasing hormone analogues was carried out with minimal side-chain protection. Catalytic transfer hydrogenation was employed for removal of NO_2 and Z-groups from Arg and <Glu respectively avoiding the use of HF and this led to good yields. An aromatic, hydrophilic amino acid, D-(*p*-hydroxyphenyl) glycine was incorporated into luteinizing hormone releasing hormone molecule along with other modifications. The agonistic as well as antagonistic activities of all the peptides have been studied.

Keywords. Fmoc-amino acid trichlorophenyl esters; solid phase synthesis; minimal side-chain protection; LHRH analogues; biological studies.

oduction

theses of the hypothalamic hormone, luteinizing hormone releasing hormone RH) and its agonists and antagonists using acid labile protecting groups like , Z, etc., for α-amino or side-chain protection generally involves final treatment anhydrous liquid hydrogen fluoride leading to contamination of the final duct with closely related impurities thereby necessitating extensive purificaiton. solid phase synthesis (SPS) of peptides, use of base labile 9-fluorenylmethyl-carbonyl (Fmoc) group for N^α-protection would allow milder conditions to be loyed during the synthesis in addition to the requirement of minimal side-chain ection and this strategy was followed for the synthesis of LHRH analogues.

survey of literature on structure-activity studies of several LHRH analogues ally and Coy, 1977; Schally, 1978; Schally *et al.*, 1979, 1980), indicates that ly potent agonists result from introduction of either aliphatic D-amino acids bulky side-chains or aromatic D-amino acids in the place of Gly^6 residue, /or by replacement of Gly^{10} residue by alkylamide (particularly ethylamide). A bination of these two modifications has synergistic effects on biological activity. modification that enabled antagonists to be potent enough to block ovulation

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reviations used: LHRH, Luteinizing hormone releasing hormone; SPS, solid phase synthesis; Fmoc, was the incorporation of aromatic D-amino acids such as D-Phe rather than alkyl D-amino acids in place of His^2 and/or Gly^6 residues. D-Trp in position 3 has been extensively used to increase the activity of antagonists. Replacing <Glu in position 1 by the D-isomer or aromatic D-amino acid causes increase in potency (Rivier and Vale, 1978). Acylated D-amino acids at this position give even better results (Channabasavaiah and Stewart, 1979). The effective dose of antagonists can further be lowered by replacement of Gly^{10} by D-Ala (Erchegui *et al.*, 1981). Taking into account all these observations, 17 LHRH analogues (table 1) involving single or multiple changes at positions 1, 2, 3, 6 and 7 were designed. In some of these modifications, an aromatic hydrophilic amino acid, D-(*p*-hydroxyphenyl)glycine (Hpg) was incorporated as this would enable us to understand the role of a phenolic group on biological activities.

Materials and methods

Synthesis

All the amino acids employed, except glycine, are of L-configuration unless otherwise specified. The purity of the final peptides was demonstrated by paper chromatography on Whatman No. 1 chromatography paper strips by ascending method using the following solvent systems:

A. n-BuOH-HOAc-H_2O (4:5:5, upper phase, v/v)
B. n-BuOH-HOAc-H_2O-pyridine (30:6:24:20, v/v)

and R_f values were expressed as R_f A and R_f B respectively. For amino acid analyses, peptides were hydrolysed with 6 N HCl at 100° for 24 h. Elemental analyses were carried out after drying the products for 12 h at 50°C under vacuum.

p-Benzyloxybenzyl alcohol resin was prepared by refluxing 4-hydroxybenzyl alcohol and Merrifield resin in the presence of NaOMe in dimethylacetamide at 100°C for 48 h (Wang, 1973; Gui-shen Lu *et al.*, 1981). Fmoc-Cl prepared according to Carpino and Han (1972, 1973) was used for the introduction of Fmoc group into all the amino acids except for Gly and D-Ala for which Fmoc-N_3 (Tessier *et al.*, 1983) was used. Fmoc-amino acids and their active esters were prepared according to Chang *et al.* (1980) and Sivanandaiah and Gurusiddappa (1984) respectively.

The protocol for the synthesis of LHRH analogues is outlined in figure 1. The first amino acid was anchored to *p*-benzyloxybenzyl alcohol resin as its Fmoc derivative using DCC in the presence of *p*-dimethylaminopyridine (Meienhofer *et al.*, 1979) and the amino acid content on the resulting resin was determined spectrophotometrically. Fmoc cleavage was effected by using the mild base 60% Et_2NH in DMF in about 3 h. The completion of deprotection and coupling was monitored by Kaiser's qualitative ninhydrin test (Kaiser *et al.*, 1970). The protected peptide was released from the resin by ammonolysis/ethylammonolysis and the peptide thus obtained was finally subjected to catalytic transfer hydrogenation (CTH) (Sivanandaiah and Gurusiddappa, 1979, 1982). The product was purified by ion-exchange chromatography using CM-Sephadex C-25 and gel filtration on Sephadex G-25 and the chromatographically pure peptides were obtained in 31–42% yield (based on the original amino acid esterified to the resin).

Fmoc-Gly + HO-CH_2-C_6H_4-OCH_2-C_6H_4-Resin

$\downarrow$ DCC and *p*-dimethylaminopyridine

Fmoc-Gly-OCH_2-C_6H_4-OCH_2-C_6H_4-resin

$\downarrow$
1) 60% Et_2NH in DMF, 180 min.
2) Coupling with 3 equiv. of appropriate Fmoc-amino acid active ester in the presence of 1 equiv. of HOBt in DMF [Fmoc-Arg(NO_2) and Fmoc-Ser (3 equiv.) were coupled in the presence of DCC (3 equiv.) and HOBt (1 equiv.) in DMF : CH_2Cl_2 (1 : 2). <Glu was incorporated as Z-<Glu-OTcp in the presence of HOBt in DMF].

Z-<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO_2)-Pro-Gly-OCH_2-C_6H_4-OCH_2-C_6H_4-Resin

$\downarrow$ NH_3-CH_3OH

Z-<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO_2)-Pro-Gly-NH_2

$\downarrow$
1) Pd black/85% HCOOH at room temperature
2) CM Sephadex C-25
3) Sephadex G-25
4) Lyophilisation

<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg-Pro-Gly-NH_2

Figure 1. Outline of the synthesis of [D-Hpg^6] LHRH.

erimental

thesis of [D-Hpg^6]LHRH (analogue II)

oc-Gly-OCH_2-C_6H_4-OCH_2-C_6H_4-Resin: Fmoc-Gly (0·99 g, 3·3 mmol) was made to ct with Hpg resin (2 g) at 0°C in the presence of dicyclohexylcarbodiimide (DCC) 8 g, 3·3 mmol) and *p*-dimethylaminopyridine (0·4 g, 3·3 mmol) for 30 min and at m temperature for 5 h. The reaction mixture was then chilled, treated with zoyl chloride (1·14 ml) and pyridine (1·02 ml) and shaken for 30 min at 0° and at room temperature. The yield was found to be 2·26 g. The Fmoc-Gly content gram of resin was estimated to be 0·41 mmol by spectrophotometric ermination of N-(9-fluorenylmethyl)piperidine.

< Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO_2)-Pro-Gly-$OCH_2C_6H_4$-OCH_2-C_6H_4-in: Fmoc-Gly-OCH_2-C_6H_4-OCH_2-C_6H_4-Resin (2 g, 0·7 mmol) was taken a horizontal solid phase vessel and peptide synthesis was carried out by the nual procedure, similar to that employed for the synthesis of [Leu^5]enkephalin anandaiah and Gurusiddappa, 1981). Fmoc-amino acid active esters (3 equiv.) in presence of 1-hydroxybenzotriazole (HOBt) (1 equiv.) was employed throughout ept for the N-terminal residue when Z-<Glu-trichlorophenyl ester (OTcp) was d. The synthesis was carried out by a stepwise single addition of appropriate ino acids, until the assemblage of the target peptide was completed. The yield found to be 2·74 g.

< Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO_2)-Pro-Gly-NH_2: The protected deeptide resin, Z-<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO_2)-Pro-Gly-OCH_2-H_4-OCH_2-C_6H_4-Resin was suspended in absolute methanol (75 ml/g) and subed to ammonolysis for the release of protected peptide amide. The crude peptide s obtained was dissolved in a minimum amount of methanol and precipitated

<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg-Pro-Gly-$NH_2 \cdot 3HOAc$: To a solution of protected. peptide, Z-<Glu-His-Trp-Ser-Tyr-D-Hpg-Leu-Arg(NO_2)-Pro-Gly-NH_2 (0·20 g, 0·15 mmol) in 85% HCOOH (2 ml) and methanol (1 ml), freshly prepared palladium black (0·15 g) was added and stirred for 2 h at room temperature. The catalyst was filtered and the solution was evaporated *in vacuo*. The residue was dissolved in methanol (2 ml) and precipitated by adding ether. The isolated solid was reprecipitated using ethanol-ethyl acetate to yield 0·155 g of the product. This crude peptide was dissolved in a minimum amount of 0·005 M ammonium acetate buffer (pH 6·9) and subjected to ion-exchange chromatography on a column (2 × 11 cm) of CM-Sephadex C-25 (Pharmacia, Sweden) using a stepwise gradient of 0·005, 0·01, 0·05, 0·075, 0·1 and 0·2 M ammonium acetate buffer (pH 6·9) with a flow rate of 60 ml/h. The column fractions were monitored at 280 nm. The fractions corresponding to 0·075 and 0·1 M buffer containing the desired peptide were pooled and lyophilised. The resulting residue was dissolved in the minimum amount of 0·5 M HOAc and the solution was subjected to gel filtration on a column (1·5 × 160 cm) of Sephadex G-25 using the same solvent for elution. Material corresponding to the central portion of the main peak was recovered by lyophilisation as a white fluffy powder. Paper chromatography on Whatman No. 1 chromatography paper strips by ascending method with *n*-BuOH-HOAc-H_2O (4 : 1 : 1) gave a single spot after spraying with Pauli, Ehrlich, or Sakaguchi reagent and no spot with ninhydrin reagent. The yield was found to be 0·072 g (overall yield 41% based on the amount of Fmoc-Gly esterified to the resin); R_f A, 0·60; R_f B, 0·69; $[\alpha]_D^{25}$ −37° (c, 1; 1% HOAc).

Analysis: $C_{68}H_{89}O_{19}N_{15}$ Calcd. C, 57·50; H, 6·2; N, 14·79
Found. C, 57·12; H, 6·01; N, 14·58%.

Amino acid analysis: Gly(l), 1·05; Pro(l), -[a]; Arg(l), 0·87; Leu(l), 0·93; Hpg(l), 1·13; Tyr(l), 1·2; Ser(l), 0·91; Trp(l), -[b]; His(l), 0·91; Glu(l), 1·04.

[a] Pro was not estimated.

[b] Trp was destroyed during acid hydrolysis.

The other 16 analogues were synthesized by following the same procedure with appropriate modifications at each stage and the protected peptide resins were subjected to ammonolysis/ethylammonolysis by using CH_3OH-NH_3/CH_3OH-anhydrous ethylamine. The crude protected peptides, after isolation, were deprotected by 85% HCOOH/Pd black and purified by following the same method employed for the purification of [D-Hpg^6]LHRH and yields of peptides are given in table 1.

Biological assay

The biological assays were performed at the Institute for Research in Reproduction, Bombay. The activities of these peptides were determined by *in vitro* anterior pituitary cell culture method. Following the method of Vale *et al.* (1972a, b), the ability to stimulate LH release (agonistic activity) and to inhibit LHRH stimulated LH release (antagonistic activity) of all the LHRH analogues were determined.

able 1. List of LHRH analogues synthesized with yields and LH-releasing activity at 10 ng ose.

eptide	Yield* (%)	LH-release in ng/ml
ontrol		1·4 1·4
tandard LHRH		45·8 38·2
D-Hpg^{2}]LHRH (I)	40·0	1·8 1·8
D-Hpg^{6}]LHRH (II)	41·0	6·7 9·8
D-Hpg^{7}]LHRH (III)	36·7	1·7 2·1
D-Phe^{2}, D-Hpg^{6}]LHRH (IV)	36·7	1·9 2·6
Tyr^{2}, D-Hpg^{6}]LHRH (V)	39·2	1·7 2·1
Tyr^{2}, D-Phe^{6}]LHRH (VI)	36·2	2·3 2·6
D-Hpg^{2}, D-Phe^{6}]LHRH (VII)	37·2	1·9 2·1
D-Phe^{1}, D-Hpg^{2}, D-Trp^{3}, Trp^{6}]LHRH (VIII)	37·9	1·9 1·4
N-Ac-D-Trp^{1}, D-Hpg^{2}, D-Phe^{6}]LHRH (IX)	38·0	2·4 1·3
N-Ac-D-Phe^{1}, D-Hpg^{2}, D-Trp^{3}, Trp^{6}]LHRH (X)	38·8	2·4 2·5
N-Ac-D-Trp^{1}, D-Hpg^{2}, D-Trp^{3,6}]LHRH (XI)	36·5	1·8 1·6
Tyr^{2}, D-Trp^{3}, D-Ala^{6,10}]LHRH (XII)	36·2	1·7 2·4
D-Hpg^{2}, D-Trp^{3}, D-Ala^{6,10}]LHRH (XIII)	41·3	1·8 1·5
D-Hpg^{7}, Pro^{9}-NHEt]LHRH (XIV)	33·3	1·9 1·4
D-Trp^{6}, D-Hpg^{7}, Pro^{9}-NHEt]LHRH (XV)	32·1	3·2 2·2
D-Hpg^{2,3}, (D-Trp-D-Hpg)^{7}, Pro^{9}-NHEt]LHRH (XVI)	34·5	2·3 2·0
D-Hpg^{2}, D-Trp^{6}, Pro^{9}-NHEt]LHRH (XVII)	32·1	1·6 2·5

Overall yield is based on the amount of amino acid esterified to the resin.

ults and discussion

thesis

e time required for the completion of coupling of each amino acid is about 1 to . The yields of the peptides are given in table 1, and the physical constants and lytical data in table 2. Amino acid analyses of all the peptides gave satisfactory ults.

Table 2. Physical constants and elemental analysis of peptides I, III to XVII.

				Elemental analysis Calcd. (found) %		
Peptide	R_fA	$[\alpha]_D^{25}$ (c, 1; 1% HOAc)	Molecular formula	C	H	N
I	0·62	−34	$C_{61}H_{83}O_{18}N_{15}$	55·72	6·33	15·98
				(55·38	6·15	15·61)
III	0·59	−34	$C_{67}H_{91}O_{20}N_{17}$	55·31	6·30	16·36
				(55·12	6·03	15·99)
IV	0·58	−34	$C_{68}H_{89}O_{18}N_{15}$	58·16	6·34	14·96
				(57·94	6·01	14·63)
V	0·62	−36	$C_{68}H_{89}O_{19}N_{15}$	57·50	6·27	14·79
				(57·09	6·05	14·49)
VI	0·64	−41	$C_{69}H_{91}O_{18}N_{15}$	58·43	6·42	14·82
				(57·98	6·12	14·70)
VII	0·59	−28	$C_{68}H_{89}O_{18}N_{15}$	58·16	6·34	14·96
				(57·89	6·09	14·69)
VIII	0·64	−36	$C_{74}H_{94}O_{17}N_{16}$	61·82	6·37	15·14
				(61·54	6·07	14·97)
IX	0·63	−30	$C_{74}H_{92}O_{16}N_{16}$	60·82	6·30	15·34
				(60·71	6·19	15·11)
X	0·69	−28	$C_{74}H_{92}O_{16}N_{16}$	60·82	6·30	15·34
				(61·02	6·25	15·15)
XI	0·71	−30	$C_{76}H_{93}O_{16}N_{17}$	60·82	6·25	15·86
				(60·23	6·24	15·69)
XII	0·56	−39	$C_{64}H_{87}O_{19}N_{14}$	56·63	6·41	14·45
				(56·43	6·18	14·24)
XIII	0·63	−33	$C_{63}H_{85}O_{19}N_{14}$	56·37	6·33	14·61
				(56·09	6·10	14·49)
XIV	0·68	−31	$C_{65}H_{89}O_{19}N_{16}$	55·80	6·41	16·02
				(55·53	6·32	15·77)
XV	0·65	−20	$C_{74}H_{96}O_{19}N_{17}$	58·17	6·33	15·58
				(57·86	6·23	15·34)
XVI	0·63	−29	$C_{73}H_{92}O_{20}N_{15}$	58·45	6·18	14·00
				(58·01	6·12	13·69)
XVII	0·67	−21	$C_{70}H_{91}O_{17}N_{15}$	59·44	6·44	14·86
				(58·97	6·24	14·68)

combination of Z for <Glu and NO_2 for Arg permitted the final deprotection of the protected peptide by CTH. As a result, the drastic treatment of the protected peptide with HF at the end of the synthesis was circumvented. Use of Fmoc group for N^α-protection and coupling by the active ester method necessitated minimal side-chain protection leading to better yields (35–40% based on the original amino acid esterified to the resin) of chromatographically pure peptides.

Biological assays

The LH-releasing activity of all the analogues (agonistic activity) *i.e.*, LH levels measured by radioimmunoassay is given in table 1. These assays indicate that these analogues possess very little LHRH activity, with [D-Hpg^6] LHRH (analogue II) having only 5% of the activity. Though, in general, replacement of the Gly^6 residue in LHRH by D-amino acids, particularly aromatic residues such as D-Phe and

D-Tyr makes the molecule agonistic, introduction of D-Hpg at this position leads to weak agonistic analogues only. The loss in activity is also observed when D-Hpg is substituted at position 2 or 7 (analogues I and III). Further, introduction of D-Hpg at position 2 or 7 in [D-Trp^6, Pro^9-NHEt] LHRH and at position 7 in [Pro^9-NHEt] LHRH (analogues XVII, XV and XIV), leads to marked decrease in potency of these peptides, which are several times more active than LHRH.

The antagonistic properties were studied by their ability to inhibit LHRH stimulated LH release by adding 10 ng of standard LHRH and an analogue at 10 ng dose to duplicate cultures. These results indicate that, at a dosage of 10 ng, all these analogues possess no significant antagonistic activity. Though analogues like [D-Phe^2, D-Trp^6] LHRH, [D-Phe^{2,6}] LHRH, are known to be good antagonists, the introduction of D-Hpg residue at position 6 in [D-Phe^2] LHRH (analogue IV) leads to a marked decrease in potency. Further, introduction of Ac-D-Trp at position 1 in [D-Hpg^2, D-Phe^6] LHRH renders the molecule inactive (analogue IX). Tyr or D-Hpg at position 2 of [D-Trp^3, D-Ala^{6,10}] LHRH does not enhance activity at 10 ng dosage (analogues XII and XIII). These results suggest that introduction of D-Hpg into LHRH molecule generally leads to the inactivity.

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lecular basis of activity of 8-azapurines in transcription processes

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Abstract. The quantum mechanical perturbation method has been utilized to study the biological activity of 8-azapurine (8-azaguanosine, 8-azaadenosine and 8-aza-2,6-diaminopurine) nucleoside antibiotics. The in-plane (hydrogen bonding) and stacking energy of 8-azapurine bases have been evaluated with nucleic acid bases and base pairs in all possible orientations. The energy values and the sites of association of analogous bases, obtained by optimization of energy values as well as the sites of association of nucleic acid bases during the transcription process have been compared. The model developed earlier for the incorporation of nucleoside analogues has been used to find out the inhibitory effects of the drug on nucleic acid and protein synthesis. It has been observed that the activity of 8-azapurines are of the following order

8-azaguanine > 8-aza-2,6-diaminopurine > 8-azaadenine

and these analogues show preference for binding near a guanine or cytosine in the chain. The results are in agreement with the experimental observations.

Keywords. Interaction energy; nucleoside analogues; transcription; incorporation and inhibition.

›duction

leosides play an important role in cellular metabolism, and any chemical
ification brings about an alteration of the nucleotide structure as a whole. A
ber of synthetic and natural nucleoside analogues with interesting biological
erties are known and some of them have already been successfully employed as
ıpeutic agents. These nucleoside analogues all share the common feature that
biological function requires an intracellular conversion to the corresponding
eotides. Investigation of the mechanism by which these nucleoside analogues
fere with cellular metabolism, therefore, requires sufficient data on interaction
nucleic acid fragments at the molecular level. The differences in the activity
een the naturally occurring bases and the modified bases are partly due to the
tural changes and partly to the electronic effects brought about by the various
ifications. One general class of modified bases is characterized by the sub-
tion of a N atom for a C–H group or *vice-versa*. The class includes the
ıpurines, 6-azapyrimidines, 3-deazapyrimidine and 7-deazaadenine. Nucleosides
-azapurines, known as one of the orthoazanucleosides, have the interesting
res that without the H atom at the ortho position, the steric behaviour of
ion of the base around the glycosyl bond is greatly reduced.

sing the model developed by Sanyal *et al.* (1981a, 1984, 1987) an attempt
been made for the first time to explain the drug action with particular
ence to pyrazolopyrimidine (Sanyal *et al.*, 1986c, 1987), pyrrolopyrimidine
yal *et al.*, 1984), 6-azapyrimidine (Ojha *et al.*, 1988) etc. In the previous papers
is series (Sanyal *et al.*, 1980a, b, 1981a, b, 1984), the interaction of some
iotic nucleosides with the DNA base pairs have been studied within the entire
of deep groove of the DNA double helix, using the electrostatic hard-sphere
el. It was shown by computing the interaction energy and taking into account

the possible sites of association, that the possibility of incorporation of the analogous molecules instead of the normal bases during transcription can be predicted. In continuation of these studies, we have extended our computation to calculate the higher terms up to second order. In the present paper an attempt has been made to investigate the biological activity of 8-azapurines (figure 1) on the basis of our earlier model (Sanyal *et al.*, 1986a, b, 1987).

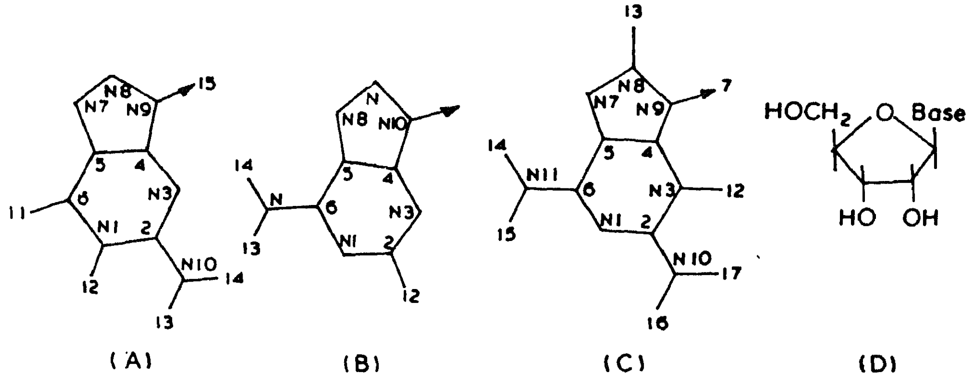

Figure 1. Chemical structure of 8-azapurines. (A), 8-Azaguanine; (B), 8-azaadenine; (C), 8-aza-2, 6-diaminopurine; (D), nucleoside.

8-Azapurines (8-azaguanine, 8-azaadenine and 8-aza-2,6-diaminopurine) are important analogues of nucleic acid constituents because of their anti-neoplastic properties. The carcinostatic activities of these compounds vary substantially *e.g.*, 8-azaguanine (Montegomery *et al.*, 1970) showing a broad range of activity while 8-aza-2,6-diaminopurine is only moderately effective against adenocarcinoma 755 (Montegomery, 1959). 8-Azaguanine gets incorporated into nucleic acids of tumors and viruses (Mitchell *et al.*, 1950; Matthew, 1953; Smith and Matthew, 1957). Substitution of 8-azaguanine for guanine in RNA is considerable while very little substitution is possible in DNA (Mandel *et al.*, 1954). 8-Azaadenine has also been studied for its antileukemic activity (Shelley and Glayton, 1973). Experimental studies on 8-azaadenine nucleoside are limited.

The conformation (Singh and Hodgson, 1974) about axocyclic (C(4)′–C(5)′) bond is *gt* and conformation about glycosidic bond (χ_{CN}) is in the intermediate range between anti and *syn* region called high anti (Prusiner *et al.*, 1973). The NMR studies on 8-azapurine in aqueous solution show that the sugar conformation is in C(3′)-endo C(2′)-endo equilibrium and the conformation about glycosyl bond is in *syn*$\rightleftharpoons$anti equilibrium (Lee *et al.*, 1975). Ludemann *et al.* (1976) observed that the *gg* conformation is preferred over *gt* and *tg* conformations. Based on PCILO computation, Saran *et al.* (1978) have suggested that 8-azapurine nucleosides having C(2′)-endo and C(3′)-endo sugar puckering exhibit the most preferred conformation *gg* for exocyclic bond. For the glycosyl torsion angle (χ_{CN}), C(2′)-endo 8-azanucleosides prefer anti whereas C(3′)-endo nucleosides prefer *syn* conformation.

Methodology

The details of the method have already been discussed in the previous papers (Sanyal *et al.*, 1980a, b, 1981a,b, 1984, 1985b, 1986a, b, c, 1987). The crystallographic cture of 8-azapurines and nucleic acid fragments have been taken from ature (Singh and Hudgson, 1974; Purnell and Hudgson, 1976; Singh and lgson, 1975; Voet and Rich, 1970). CNDO/2 method (Pople and Beveridge,)) has been used to compute the monopole and dipole components at the nic centres. These molecular charges have been used to calculate the interaction rgies. The total energy may be expressed as a sum of various components

$$E_{\mathrm{tot}} = E_{\mathrm{el}} + E_{\mathrm{pol}} + E_{\mathrm{disp}} + E_{\mathrm{rep}}$$

re E_{tot} stands for the total interaction energy while E_{el}, E_{pol}, E_{disp} and represent the electrostatic, polarization, dispersion and repulsion energies pectively. The electrostatic component is the resultant of the sum of interactions ween various atomic multipole *i.e.*, monopole-monopole, monopole-dipole and ole-dipole. The details of the formula used may be found in the works of Caillet Claverie (1975), Claverie (1978), Vasanth Kumar and Govil (1982) and Sanyal *l.* (1986b, c, 1987). The minimization method (Sanyal *et al.*, 1986b, c, 1987) has n used to obtain minimum energy configuration.

Computation was carried out on CDC-Cyber 170 Computer at the National nputer Centre, Tata Institute of Fundamental Research, Bombay.

lane interaction energy

in-plane interactions can be grouped into two parts, one represents the raction with bases while the other represents interaction with base pairs.

ier energy

the present case, since we are interested in the transcription and replication cess and the stability of nucleic acids, the pairing energy corresponding to the ition of Watson–Crick pair has been calculated. Table 1 shows the pairing rgies of 8-azapurine with pyrimidine and the corresponding configurations are wn in figure 2. The pairing energies of nucleic acid bases have already been cussed in detail (Sanyal *et al.*, 1986a, b, c, 1987). The complex formed by zaguanine (azaG) is stabler than the other complexes. The energy values

le 1. Dimer energy: Hydrogen bonding energy (in kcal/mol) of 8-azapurines with pyrimidines.

llogue	Complex formed		E_{el}	E_{pol}	E_{disp}	E_{rep}	E_{tot}
zaguanine (azaG)	azaG:C		−21·02	−3.23	−7.75	9.59	−22.41
	azaG:U		−12·13	−2·85	−5·71	6·58	−14·10
	azaG:T		−11·77	−2·83	−5·87	6·59	−13·88
zaadenine (azaA)	azaA:C		−8·72	−1·31	−6·09	6·65	−9·47
	azaA:U		−7·50	−0·92	−5·83	5·63	−8·62
	azaA:T		−7·38	−0·91	−5·90	5·63	−8·56
za-2,6-diaminopurine DAP)	ADAP:U	I	−8·89	−1·36	−7·17	7·17	−10·25
		II	−8·69	−1·39	−7·25	7·31	−10·02
	ADAP:T	I	−8·76	−1·36	−7·25	7·18	−10·19
		II	−8·74	−1·39	−7·31	7·32	−10·13

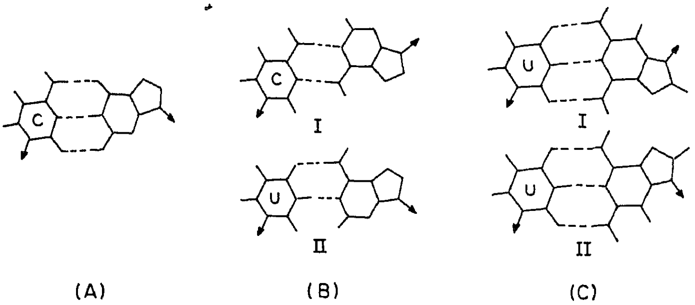

Figure 2. Dimer complexes of 8-azapurines with nucleic acid bases. (A), 8-Azaguanine; (B), 8-azaadenine; (C), 8-aza-2, 6-diaminopurine.

corresponding to azaG : U/T complexes are considerable corresponding to the energies of A : U/T pairs but they distort the nucleic acid chain and tend to restrict the elongation process. 8-Azaadenine (azaA) forms Watson–Crick pairs with U/T with the energy values comparable to A : U/T pair (−8·52/−8·49 kcal/mol) and it forms a distorted complex (figure 2) with cytosine. The complex formed by 8-aza-2, 6-diominopurine have the energy values greater than those for the standard dimer energy for A : U/T base pairs. In figure 2, one complex will distort the chain and stops the growth of the nucleic acid, while the other is helpful in template recognition during replication process.

For all the complexes, the electrostatic component is dominant and primarily responsible for the binding of the two molecules in the positions shown in figure 2, while the polarization and dispersion terms reduce the effect of repulsion term and are eventually useful for searching the minimum energy positions.

Trimer energy

The total association energy of the analogue with the nucleic acid base pairs along with the various components of energy in the minimum energy position are shown in table 2. The minimum energy configurations corresponding to these energies are given in figure 3. The comparative studies of the site of interaction of these complexes with the standard complexes are shown in figure 4. The arrows represent the glycosyl bond of the 8-azapurine nucleoside in different configurations. The head of the arrow points towards the (Cl′) atom of sugar moity, while the tail represents the N9 atom of the azapurine base. The arrows with star-marks represent the recommended site of association in transcription process of nucleic acid bases (Sanyal *et al.*, 1986a, b, c, 1987).

Complex I is formed by the interaction of 8-azaguanine from the deep groove side of G–C base pair which bears the energy value −22·42 kcal/mol. Complexes II and III are formed by A–U and A–T base pairs. The energy values with A–U pair are significant while the energy values with A–T pair are comparable to the (A–U)–A and (A–T)–A complexes (Sanyal *et al.*, 1986a, b, c, 1987). In figure 3A, only the complexes with A–U pairs are shown because the position of enterant analogous base is same with both A–U and A–T pairs.

2. Trimer energy: Hydrogen bonding energy (in kcal/mol) of 8-azapurines with nucleic acid base

nt base	Base pair	E_{el}	E_{pol}	E_{disp}	E_{rep}	E_{tot}	Complex
	G–C	−20·74	−3·44	−7·93	9·69	−22·42	I
	A–U	−9·00	−2·50	−8·70	9·15	−11·07	II
		−3·36	−1·32	−6·00	4·91	−5·75	III
	A–T	−5·59	−1·67	−7·69	6·03	−8·83	II
		−3·50	−1·34	−6·02	4·94	−5·92	III
	A–U	−4·45	−0·89	−6·28	5·91	−5·71	IV
		−2·40	−0·86	−5·71	5·45	−3·61	V
	A–T	−4·34	−0·86	−6·51	5·91	−5·79	IV
		−2·54	−0·87	−6·75	5·45	−3·71	V
	G–C	−8·99	−1·83	−5·64	5·74	−10·63	VI
		−0·72	−1·31	−5·06	3·32	−3·77	VII
	G–C	−7·65	−1·33	−7·19	6·94	−9·23	VIII
		0·40	−1·96	−6·50	4·98	−3·07	IX
	A–U	−4·58	−1·45	−7·72	6·71	−7·05	X
		−3·03	−1·23	−7·36	6·76	−4·86	XI

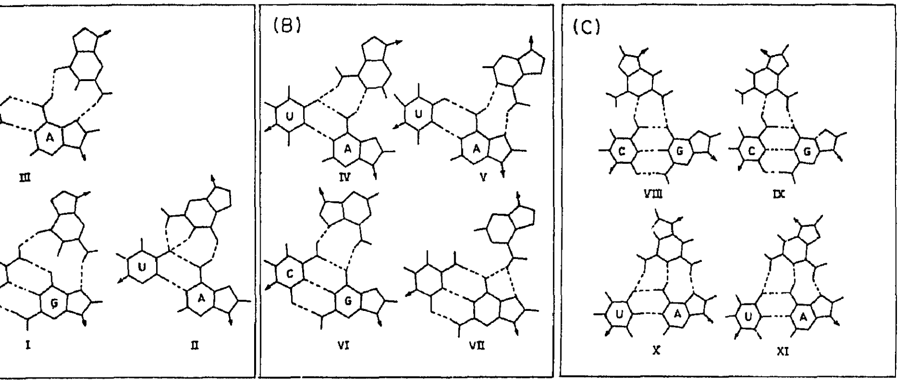

Figure 3. Minimum energy configurations formed by 8-azapurine with nucleic acid bases. (A), 8-Azaguanine; (B), 8-azaadenine; (C), 8-aza-2, 6-diaminopurine.

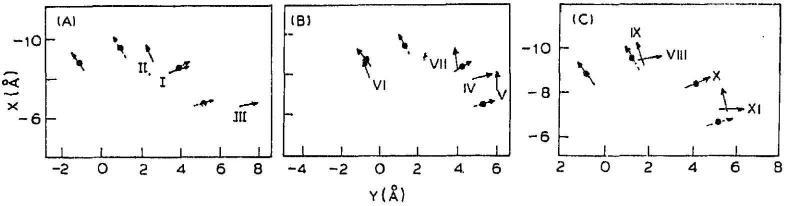

Figure 4. Arrow diagram corresponding to the sugar ends of the enterant analogue in the minimum energy configuration of base pair ,interaction. The arrows with asterisk represent the recommended site of interaction. The origin is considered at the N1 atom of purine in the base pair and the X-axis along the N1-C2 bond. (A), 8-Azaguanine; (B), 8-azaadenine; (C), 8-aza-2, 6-diaminopurine.

y value for complex IV is comparable to the energy value of (A–U/T)–A lex while the other complexes have lesser energy values than the mended energies (Sanyal *et al.*, 1986a, b, c, 1987).

Two complexes VIII and IX are formed by interaction of 8-aza 2,6 diaminopurine base from the deep groove side of G–C base pair, as shown in table 2 and figures 3C and 4C. The two complexes bear the energy values −9·25 kcal/mol and −3·07 kcal/mol respectively. The magnitude of energy values for these complexes are smaller than (G–C)–G and (G–C)–C complexes recommended for incorporation of nucleosides during transcription process (Sanyal *et al.*, 1986a, b, 1987). The trimer complexes formed by A–U pair are X and XI with energy values −7·05 kcal/mol and 4·86 kcal/mol respectively are reported in table 2, while the corresponding minimum energy configurations are shown in figure 3C. These energy values are comparable to the energy values for (A–U)–A and (A–T)–A complexes (Sanyal *et al.*, 1986a, b, c, 1987).

The stability of the complexes for the 8-azapurines can be represented as

I > II > III for 8-azaguanine
VI > IV > VII > V for 8-azaadenine

and

VIII > X > XI > IX for 8-aza-2,6-diaminopurine

and the electrostatic terms are primarily responsible for their binding.

It is clear from figure 4A that the site of interaction corresponding to complex I is the same as that recommended for transcription. The corresponding energy value is more than the energy value of (G–C)–G complex (−20·91 kcal/mol). The complex II has energy value more than the (A–U/T)–A complexes (−5·01/−4·42 kcal/mol), but the site of association is displaced from the recommended site of association and also has an angular deviation. The complex III has energy value similar to the recommended energy value but the site of association is again displaced from the recommended site. From this discussion it is clear that, only complex I is suitable for transcription energywise and also in terms of spatial position.

In case of 8-azaadenine there are four complexes IV–VII, two (IV, V) for interaction with (A–U)/(A–T) pairs and the other two (VI, VII) for interaction with the G–C pair. It is found that the complex IV with energy value −5·71/−5·79 kcal/mol is suitable for binding in the deep groove during the transcription process. Complex V with A–U/A–T pairs having energy value −3·61/−3·71 kcal/mol has lower energy value than the recommended energy values. The site of interactions for these complexes are illustrated in figure 4B. Complex IV is slightly displaced from the star-marked arrow (which represent the recommended site), while the arrow corresponding to complex V is appreciably displaced from the recommended site and also has an angular deviation. A comparison of energy values of VI and VII complexes (table 2) and the site of association shown in figure 4C with recommended (star-marked) sites, show that they have energy values lower than the energy values for the (G–C)–G complex and therefore, binding is not possible. The arrows corresponding to these complexes (VI and VII) are considerably displaced and deviated in varying degrees with respect to recommended site. Therefore, it is inferred that the binding of 8-azaadenine is not possible at the probable sites of association, but their binding as represented by complex IV with A–U pair and complex VI with G–C pair cannot be totally ruled out. Since the association site for complex IV is displaced, it may be postulated that it may attain the predicted site with minor decrease in their binding energy. In other words, it requires some ional amount of energy to place the complex at the specified site (Sanyal *et al.*, a, b, c, 1987). This additional energy can be made available in the system due to ersion of ATP to AMP and also due to the presence of some enzymes (Stent, Watson 1970; Saenger 1984). Saran *et al.* (1978) have suggested that for adenine the energy difference between its conformation from *gg* to *gt* and *tg* for -C(5′) bond is 1 kcal/mol, while it requires 0·5–1 kcal/mol energy to change the rmation from anti → *syn* for χ_{CN}. The binding through these complexes res appreciable additional energy which may easily alter the conformation anti → *syn*. This restricts the incorporation of 8-azaadenine during cription process.

za-2, 6-diaminopurine forms 4 complexes VIII–XI, two (VIII and IX) with pair and other two (X and XI) complexes with A–U pairs. As shown in figure he complex VIII is widely devited and displaced from the recommended site the complex IX is slightly displaced from the standard position (arrows with isk). The energy value for (G–C)–ADAP is less than the recommended energy s for (G–C)–G and (G–C)–C complexes as reported earlier (Sanyal *et al.*, a, b, c, 1987). It may be inferred that none of the complexes with G–C pair y the condition for the incorporation of the analogue during transcription ss. The spatial position of complex X (figure 4C) is slightly displaced, while is very little deviation from the recommended site of association. The complex appreciably displaced and deviated from the standard site of association. This lex may be displaced to the recommended site. In this process some of the iation energy has to be reduced from its earlier value (reported in table 2) n is greater than the recommended energy for (A–U)–A complex. Therefore, in ic conditions the binding through the complex X is possible during the cription process.

e conformational requirement for the incorporation of analogue is *gg* and anti yal *et al.*, 1981a, 1984, 1986a, b, c, 1987; Ojha *et al.*, 1988). As discussed r, anti⇌*syn* equilibrium exists in 8-azaguanine, which permits the incorpora- of 8-azaguanine. It is therefore, inferred that 8-azaguanosine can effectively itute for guanosine during transcription process while the incorporation of adenine is not possible. However, 8-aza-2,6-diominopurine may incorporate in hain through complex X only to some extent. The incorporation of nucleoside ogues for normal bases in the RNA chain, produces miscoding in the nucleic chain. As a result, the inhibition of protein synthesis takes place.

vin (1963) observed that a major amount of 8-azaguanine is incorporated into RNA, and the analysis of the tRNA containing 8-azaguanine revealed that only ine residues are replaced by the analogue and that the nucleotide sequence is otherwise altered (Weinstein and Grunberger, 1965). This is in agreement with results. This analogue containing tRNA does, however, appear to function ally despite the fact that one might predict apriori that slight alteration in the ture of tRNA might alter the specificity so that miscoding would result. nstein and Grunberger, 1965; Levin, 1965). This is in agreement with our ts, as discussed earlier, due to incorporation of 8-azaguanine in place of ine resulting in the change of codons. In the same way, it is easy to explain our results that 8-azaguanine is incorporated in to mRNA as observed

RNA (Otaka *et al.*, 1961) and accompanying rapid decrease in protein synthesi (Mandel, 1958). Roy *et al.* (1961) have suggested that since the mRNA and tRN are apparently functional, the inhibition of nucleic acid is not by the 8-azaguanin polyphosphate, but due to other factors. The binding of the analogue in th presence of enzymes (or other suitable experimental conditions), when some extr source of energy is available, indicate that it can stop the growth of the templat (due to conversion of anti → *syn* in the presence of energy) resulting in the inhibitio of nucleic acid synthesis (through complex II). The use of 8-azaadenosine as anti leukemic is due to its binding in the template in the presence of required energ resulting in the inhibition of the growth of the nucleic acid template. The effectiveness of 8-aza-2, 6-diaminopurine against adenocarcinoma 755 (Montegomery, 195 is thus explained (through inhibition of DNA, RNA or protein synthesis).

Stacking energy

The stacking energy of 8-azapurines with nucleic acid bases (G, C, A, U and T) ar shown in table 3 while their corresponding minimum energy configurations ar shown in figure 5. The relative effect of the energy components (electrostati polarization, dispersion and repulsion) are given in table 3. The stacking energ represent the stability of the stacked complexes. One can conclude that the stabilit of these complexes are in the following order for 8-azapurines:

$$\begin{pmatrix}\text{azaG}\\ \text{G}\end{pmatrix} > \begin{pmatrix}\text{azaG}\\ \text{C}\end{pmatrix} > \begin{pmatrix}\text{azaG}\\ \text{A}\end{pmatrix} > \begin{pmatrix}\text{azaG}\\ \text{U}\end{pmatrix} > \begin{pmatrix}\text{azaG}\\ \text{T}\end{pmatrix}$$

for 8-azaguanine,

$$\begin{pmatrix}\text{azaA}\\ \text{C}\end{pmatrix} > \begin{pmatrix}\text{azaA}\\ \text{G}\end{pmatrix} > \begin{pmatrix}\text{azaA}\\ \text{U}\end{pmatrix} > \begin{pmatrix}\text{azaA}\\ \text{T}\end{pmatrix} > \begin{pmatrix}\text{azaA}\\ \text{A}\end{pmatrix}$$

Table 3. Stacking energy (in kcal/mol) of 8-azapurine with nucleic acid bases.

Enterant analogue	Enter-acting base	E_{el}	E_{pol}	E_{disp}	E_{rep}	E_{tot}
azaG	G	−14·32	−3·32	−6·90	3·22	−21·32
	C	−9·83	−2·59	−5·20	1·91	−15·70
	A	−6·71	−1·57	−7·16	3·42	−12·02
	U	−4·15	−1·92	−6·41	2·19	−10·29
	T	−4·29	−1·71	−5·45	1·42	−10·23
azaA	G	−7·07	−1·58	−7·92	4·46	−12·11
	C	−7·49	−1·45	−6·65	3·26	−12·33
	A	−3·80	−0·51	−7·25	3·40	−8·16
	U	−5·11	−0·93	−5·68	2·43	−9·29
	T	−4·78	−0·96	−5·91	2·54	−9·10
ADAP	G	−14·21	−3·95	−14·48	10·47	−22·14
	C	−15·50	−5·30	−10·41	6·52	−24·70
	A	−9·26	−2·44	−11·29	6·74	−16·25
	U	−7·61	−3·00	−11·41	7·46	−14·56
	T	−8·60	−4·04	−10·35	6·24	−16·76

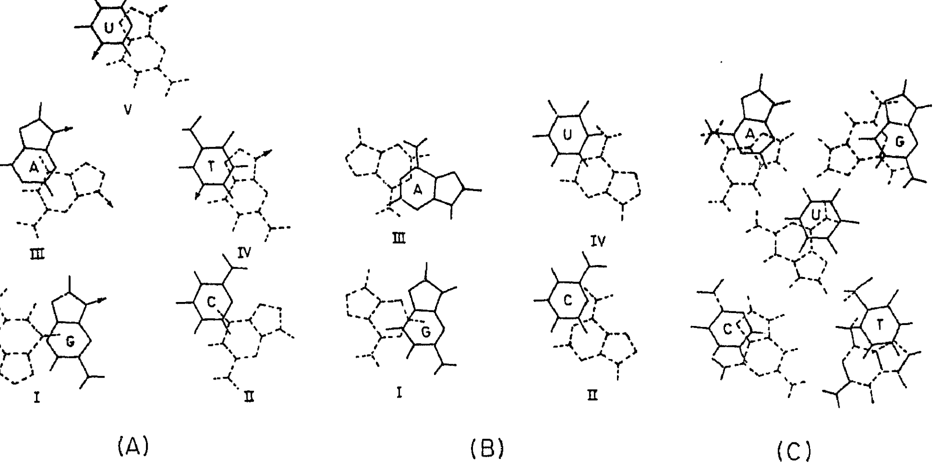

Figure 5. Stacked complexes of 8-azapurine. (A), 8-Azaguanine; (B), 8-azaadenine; (C), 8-aza-2,6-diaminopurine.

r 8-azaadenine and

$$\begin{pmatrix} \text{ADAP} \\ \text{C} \end{pmatrix} > \begin{pmatrix} \text{ADAP} \\ \text{G} \end{pmatrix} > \begin{pmatrix} \text{ADAP} \\ \text{T} \end{pmatrix} > \begin{pmatrix} \text{ADAP} \\ \text{U} \end{pmatrix} > \begin{pmatrix} \text{ADAP} \\ \text{A} \end{pmatrix}$$

r 8-aza-2,6-diaminopurine.

It is inferred that 8-azapurines prefer to bind in the neighbourhood of the dipolar ses guanine or cytosine during the template recognition. The stacking energy nsiderations and the conformational requirements of the nucleotides indicate that e selection of the specific site of these analogues for the binding near guanine or tosine depends on the selection of the particular chain of DNA on which the new mplate is formed in the transcription process.

onclusion

e interaction energy studies on 8-azapurines show that 8-azaguanine can easily incorporated in the transcription process through configuration I. The corporation of 8-azaguanosine in the RNA chain, for guanosine, alters the ecificity so that miscoding results. The production of abnormal RNA due to its corporation rapidly decreases the rate of protein synthesis which is in agreement th the experimental results. 8-Azaadenosine cannot be incorporated in the RNA ain but it may bind during the elongation process and stop the growth. 8-Aza-2, diaminopurines are moderately incorporated in the chain and binds during the ongation process to inhibit the protein and nucleic acid synthesis. 8-Azapurine efers to bind near guanine or cytosine and selection of these complexes depends the consideration of the template. These properties are perhaps responsible for e carcinostatic activities of the drugs. This study offers a mechanism for the tivity of the nucleoside antibiotic. The activity of 8-azapurines are of the following der

8-azaguanine > 8-aza-2,6-diaminopurine > 8-azaadenine.

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ect of experimentally induced thyrotoxicosis on oxidative energy abolism in rat heart mitochondria

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Abstract. Treatment of rats with T_3 resulted in a significant decrease in body weight, while the heart weight increased. T_4 treatment had less marked effect on body weights but resulted in decreased heart weights. Serum T_4 levels decreased significantly with simultaneous increase of T_3 level following T_3 treatment, whereas with T_4 treatment, levels of both T_4 and T_3 increased in the serum. Low doses of T_3 (0·5 μg) caused decrease in mitochondrial protein content while high dose of T_4 (1 μg), caused significant increase in mitochondrial mass. The state 3 respiration rates were significantly depressed following T_3 and T_4 treatments, in a substrate specific manner with the effects being more pronounced with T_3; these responses with T_4 were dose-dependent for succinate and ascorbate + N,N,N′,N′-tetramethyl-*p*-phenylenediamine. State 4 respiration rates also exhibited similar corresponding changes. ADP/O ratios were not changed but ADP-phosphorylation rates were decreased significantly particularly so with the T_3-treated animals. Treatment with T_3 also resulted in lowering of intramitochondrial cytochrome contents. Similar effects were seen also with higher doses of T_4. The results thus indicate that T_3- and T_4-thyrotoxicosis results in impaired energy metabolism in heart mitochondria.

Keywords. Thyrotoxicosis; heart mitochondria; energy metabolism.

oduction

e of thyroid hormones in regulation of mitochondrial energy metabolism and r energy-linked functions and on the basal metabolic rate (BMR) is now well blished (Tata *et al.*, 1963; Tata, 1964; Gustafsson *et al.*, 1965; Oppenheimer *l.*, 1976; Oppenheimer, 1979; Bernal and De Groot, 1980). Deficiency of thyroid nones causes decrease in metabolism with concomitant lowering of the basal abolic rate (Tata, 1964). Treatment of hypothyroid animals with thyroid mones restores these activities to normal or near normal levels, which is also ompanied by increase in the concentrations of specific respiratory components 1 as cytochromes and protein synthesis (Tata *et al.*, 1963; Tata, 1964; Gustafsson *l.*, 1965; Katyare *et al.*, 1970).

hyrotoxicosis—a condition of excessive levels of thyroid hormones—on the er hand, increases BMR and causes fast pulse (tachycardia), anxiety, palpitation, eased sweating, loss of weight, general muscular weakness, elevation of

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reviations used: BMR, Basal metabolic rate; MOPS, 3-(N-morpholino)propanesulfonic acid; TMPD, , N′,N′-tetramethyl-*p*-phenylenediamine; RIA, radioimmunoassay; RCR, respiratory control ratios; 3,3′,5-triiodo-L-thyronine; T_4, L-thyroxine.

lysosomal cathepsin D activity and uncoupling of oxidative phosphorylation, which results in hyperthermia (Williams and Bakke, 1962; Johansen *et al.*, 1978; Irvine *et al.*, 1979; De Visscher and Burger, 1980; Jabbari and Huot, 1980; Satav and Katyare, 1981, 1982). Many reports are available on the effects of thyrotoxicosis on energy metabolism in mitochondria from tissues such as liver, kidney, brain and skeletal muscle (Maley and Lardy, 1953; Hoch and Lipmann, 1954; Dow, 1967; Stocker *et al.*, 1968; Winder *et al.*, 1975; Winder and Holloszy, 1977; Satav and Katyare, 1981, 1982). It has been shown that excessive levels of thyroid hormones affect the rates of coupled phosphorylation, ADP/O ratios, cytochrome contents and ATPase activity in mitochondria (Winder *et al.*, 1975; Winder and Holloszy, 1977; Satav and Katyare, 1982).

The effects of thyroid hormones on energy metabolism in the heart are not well understood. Tachycardia associated with thyrotoxicosis is accompanied by persistent atrial fibrilation resulting in transient episodes of arrhythmia (Hoffman and Lowery, 1960). Recent studies indicate that congestive heart failure can be produced in experimental animals by administering thyroxine (Shapiro *et al.*, 1975) and that infants with neonatal thyrotoxicosis without underlying cardiac disease may develop congestive heart failure (Shapiro *et al.*, 1975). The frequency of mitral valve prolapse is twice as much in hyperthyroid than in euthyroid individuals (Channide *et al.*, 1981).

The energy metabolism of the heart relates essentially to the contractile mechanisms of the myofibrils and oxidative phosphorylation in the mitochondria (Mela-Riker and Bukoski, 1985). In the present study an attempt has therefore been made to assess the effect of thyrotoxicosis on energy metabolism in heart by studying mitochondrial oxidative phosphorylation using experimental animal model. Results of the present studies together with those on altered membrane structure-function relationships (Dave *et al.*, 1989) have helped in illustrating and explaining the underlying biochemical lesions in heart pathology.

Materials and methods

Chemicals

Sodium salts of succinic acid, L-glutamic acid and ascorbic acid as well as 3,3′,5-triiodo-L-thyronine (T_3), L-thyroxine (T_4), ADP, rotenone, Triton X-100, 3-(N-morpholino) propanesulfonic acid (MOPS), EDTA, EGTA and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. N,N,N′N′-tetramethyl-*p*-phenylenediamine (TMPD) was from British Drug Houses, Poole, Dorset, UK. All other chemicals used were of analytical-reagent grade. Radioimmunoassay (RIA) kits for thyroxine (code RIA-K5) and triiodothyronine (code RIA-K_4) were obtained from the Radiopharmaceuticals Division of Bhabha Atomic Research Centre, Bombay.

Animals

y weight (dissolved in 0·9% NaCl solution containing 5 mM NaOH) per day for
ys (Carter *et al.*, 1981, 1982; Satav and Katyare, 1981, 1982). The animals were
d on the 5th day. Control animals received the corresponding amounts of saline
cle. Concentrations of the hormones were adjusted such that the animals
ived 100 μl of hormone solution or saline vehicle per 100 g body weight. A
rd of initial and final body weights together with heart weight of both normal
thyrotoxic animals was maintained.

m T_3 and T_4

m samples were collected from control and thyrotoxic animals on the fifth day
e treatment and the contents of T_4 and T_3 in the sera were determined by RIA.

tion of mitochondria

tion of rat heart mitochondria was carried out essentially by the procedure
ribed earlier (Doussiere *et al.*, 1984; Katyare, S. S., Rajan, R. R. and
alswamy, U. V., unpublished results). The rats were killed by decapitation and
hearts were quickly removed and placed in beakers containing chilled isolation
ium (0–4°C), consisting of 0·225 M mannitol, 0·075 M sucrose and 1 mM
A, pH 7·4. The tissue was washed repeatedly with the isolation medium to free
om blood and was minced into small pieces (1·5 × 1·5 mm) and homogenized in
ss teflon Potter-Elvehjem homogenizer (wall clearance 0·18 mm) giving two up
down strokes. The homogenate (10% w/v) was centrifuged at 700 *g* for 10 min
Sorvall RC5-C refrigerated centrifuge (0–4°C) to sediment nuclei and cell debris.
supernatant was subjected to a further centrifugation at 10,000 *g* for 10 min to
nent mitochondria. The mitochondrial pellet was washed once. Finally, the
chondrial pellet obtained from 1 g of tissue was suspended in 1 ml of isolation
um and was used for the study of oxidative phosphorylation and measurement
tochrome contents. All operations were carried out at 0–4°C.

ative phosphorylation

surements of coupled phosphorylation were carried out at 25°C with a Clark
oxygen electrode essentially as described by Katyare *et al.* (1977). The medium
total volume of 1·3 ml) contained 225 mM sucrose, 20 mM KCl, 1 mM EGTA,
M MOPS buffer (pH 7·4), 5 mM potassium phosphate buffer pH 7·4 and
oximately 1 mg of mitochondrial protein. Concentrations of the substrates used
: glutamate (10 mM), pyruvate + malate (10 + 1 mM), succinate (10 mM) and
rbate (10 mM) plus TMPD (0·1 mM); with succinate and ascorbate + TMPD,
I rotenone was also included in the reaction medium. About 150–250 nmol of
P in 10–20 μl were added, and the rates of respiration in the presence of ADP
e 3) and after its depletion (state 4) were recorded. ADP/O ratios and the ADP-
phorylation rates were calculated as described (Ferreira and Gil, 1984; Katyare
., 1977).

Mitochondrial cytochrome contents

Approximately 6–10 mg of mitochondrial proteins were solubilized using freshly prepared Triton X-100 solution at a final concentration of 0·1% and the volume was made up to 6 ml with isolation medium in which 10 mM potassium phosphate buffer pH 7·4 was also included. The difference spectra of dithionite-reduced minus ferricyanide-oxidized cytochromes were recorded as described by Satav and Katyare (1982). Contents of intra-mitochondrial cytochromes were calculated using the wavelength pairs and extinction coefficients for cytochromes aa_3, b and $c+c_1$, respectively, as described (Satav and Katyare, 1982).

Protein was estimated by the method of Lowry *et al.* (1951).

Results

The effects of T_3- and T_4-induced thyrotoxicosis on the body weights of the animals and heart weight are summarised in table 1. It is evident that treatment with T_3 brought about significant decrease in the body weights (17·8 and 28·0% decrease respectively for two doses employed). Treatment with T_4 was less effective and resulted in 8·5 and 16·7% decrease respectively with the two doses. The results thus emphasize the catabolic role played by T_3 in the experimental thyrotoxicosis. Similar findings have been reported by earlier research workers (Winder *et al.*, 1975; Satav and Katyare, 1982; Cote' and Boulet, 1985).

Table 1. Effect of thyrotoxicosis on body weight and heart weight.

		Heart weight	
Treatment	Body weight (g)	(g)	Body weight (%)
Control (12)	250·4 ± 3·3	0·863 ± 0·011	0·344 ± 0·006
0·5 μg T_3 (10)	205·9 ± 5·3***	1·149 ± 0·039***	0·556 ± 0·016***
1·0 μg T_3 (10)	180·0 ± 2·1***	0·990 ± 0·010***	0·551 ± 0·008***
0·5 μg T_4 (10)	229·1 ± 7·0**	0·878 ± 0·020^{NS}	0·385 ± 0·007***
1·0 μg T_4 (19)	208·7 ± 4·3***	0·679 ± 0·016***	0·326 ± 0·005*

Animals were given T_3 or T_4 (dissolved in 0·9% NaCl containing 5 mM NaOH) in doses indicated/g body weight subcutaneously for 4 consecutive days.
Results are given as means ± SEM of the number of observations indicated in the parentheses.
$^*P < 0·05$, $^{**}P < 0·02$ and $^{***}P < 0·001$ compared with control.
NS, Not significant.

Thyrotoxicosis also brought about marked changes in the heart weight in these animals (table 1). Thus treatment with T_3 resulted in cardiac hypertrophy and the absolute weight of the heart tissue increased by 15–33% with the lower dose of T_3 being more effective than the higher dose. The effects of T_4 treatment were less marked. Cardiac hypertrophy in rabbits receiving repeated injections of T_4 has been reported by Sinha *et al.* (1982).

The data on the content of T_4 and T_3 in the serum are given in table 2. It may be noticed that treatment with T_3 brought about a 50–57% decrease in serum T_4 levels. Lower dose of T_3 (0·5 μg) resulted in a small but reproducible increase in

Table 2. Effect of experimental thyrotoxicosis on serum T_3 and T_4 levels.

Treatment	T_4 (μg/dl)	T_3 (ng/dl)
Control	6·80 ± 0·81	120·4 ± 4·3
0·5 μg T_3	3·40 ± 0·32*	134·0 ± 1·87**
1·0 μg T_3	2·94 ± 0·14***	1560·0 ± 67·82****
0·5 μg T_4	9·40 ± 1·10^{NS}	752·0 ± 14·97****
1·0 μg T_4	15·70 ± 1·03****	1100·0 ± 57·01****

Experimental details are as given in table 1.

Results are given as mean ± SEM of 5 independent observations.

$^{*}P<0{\cdot}01$, $^{**}P<0{\cdot}02$, $^{***}P<0{\cdot}002$ and $^{****}P<0{\cdot}001$ compared with control.

NS, Not significant.

serum T_3 levels (11% increase) while at the higher dose of 1 μg, the increase in serum T_3 levels was 13-fold. Levels of serum T_4 increased steadily with increasing doses of injected T_4 and amounted to 38 and 130% increase at the two doses employed. Serum T_3 levels also increased by 6–9-fold under these conditions.

The effect of thyrotoxicosis on protein content in the heart and the yield of mitochondrial proteins was next examined. These results are given in table 3. It can be seen that both T_3 as well as T_4 treatment caused significant increase in the protein content in the heart (23–86% increase) with only marginal changes in the yield of mitochondrial proteins except in the case of higher doses of T_4 where this value increased by almost 75%.

Table 3. Effect of thyrotoxicosis on protein content and mitochondrial yield in rat heart.

Treatment	Tissue protein content (mg/g tissue)	Yield of mitochondrial protein (mg/g tissue)
Control (4)	135 ± 9	10·0 ± 0·6
0·5 μg T_3 (4)	182 ± 6*	7·2 ± 0·6**
1·0 μg T_3 (4)	182 ± 3***	9·0 ± 0·8^{NS}
0·5 μg T_4 (5)	167 ± 2**	8·6 ± 0·4^{NS}
1·0 μg T_4 (5)	251 ± 15****	18·1 ± 0·8****

Experimental details are as given in table 1.

Results are given as mean ± SEM of number of observations indicated in the parentheses.

$^{*}P<0{\cdot}01$, $^{**}P<0{\cdot}02$, $^{***}P<0{\cdot}002$ and $^{****}P<0{\cdot}001$ compared with control.

NS, Not significant.

The results on effects of thyrotoxicosis on oxidative phosphorylation are given in tables 4–7. The data in table 4 summarize the effects of thyrotoxicosis on oxidative phosphorylation in rat heart mitochondria with glutamate as the substrate. It is evident that T_3 treatment resulted in approximately 70–76% decrease in the state 3

Table 4. Effect of thyrotoxicosis on oxidative phosphorylation in rat heart mitochondria with glutamate as substrate.

Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		ADP-phosphorylation rate (nmol/min/mg protein)
		+ADP	−ADP	
Control (8)	3·0±0·12	72·8±5·0	9·8±1·2	424·0±17·1
0·5 μg T_3 (8)	2·6±0·11	17·4±1·3***	7·6±0·6$^{\mathrm{NS}}$	95·4±3·3***
1·0 μg T_3 (8)	3·1±0·05	21·6±1·3***	5·6±0·3*	133·2±12·1***
0·5 μg T_4 (10)	3·1±0·09	73·4±4·6$^{\mathrm{NS}}$	8·5±1·2$^{\mathrm{NS}}$	448·6±19·8$^{\mathrm{NS}}$
1·0 μg T_4 (8)	3·1±0·08	48·8±5·3*	4·6±0·6**	294·6±27·8**

Measurements of respiration rates were carried out at 25°C with a Clark-type oxygen electrode, employing a medium (total volume 1·3 ml) containing 225 mM sucrose, 20 mM KCl, 1 mM EGTA, 10 mM MOPS, pH 7·4 and 5 mM potassium phosphate buffer, pH 7·4. Calculations of state 3 and state 4 respiration rates, ADP/O ratio and ADP-phosphorylation rates were as described in the text.

Results are given as mean±SEM of the number of observations indicated in the parentheses.

*$P<0·01$, **$P<0·002$ and ***$P<0·001$ compared with control.

NS, Not significant.

Table 5. Effect of thyrotoxicosis on oxidative phosphorylation in rat heart mitochondria using pyruvate + malate as the substrate.

Treatment	ADP/O	Respiration rate (nmol O_2/min/mg protein)		ADP-phosphorylation rate (nmol/min/mg protein)
		+ADP	−ADP	
Control (10)	3·0±0·10	105·1±5·9	19·1±1·1	630·9±42·0
0·5 μg T_3 (8)	3·1±0·07	48·3±3·1***	13·8±0·2***	297·0±22·9***
1·0 μg T_3 (8)	3·3±0·06	56·0±2·1***	10·0±0·5***	371·4±20·4***
0·5 μg T_4 (10)	3·0±0·07	74·0±4·1***	12·5±0·9***	441·6±29·7**
1·0 μg T_4 (8)	3·0±0·06	76·1±3·3***	11·8±0·8***	457·7±23·6*

Experimental details are as described in tables 1 and 4.

Results are given as mean±SEM of the number of observations indicated in the parentheses.

*$P<0·01$, **$P<0·002$ and ***$P<0·001$ compared with control.

NS, Not significant.

dose of 1 μg with a 33% decrease in the state 3 respiration rate. The state 4 respiration rates also decreased correspondingly but to a lesser extent following T_3 treatment so that the values of respiratory control ratios (RCR) were low in T_3-treated rats. By contrast, these values were somewhat higher for T_4-treated animals (data not given). The ADP/O ratios were in norml range but ADP-phosphorylation rates decreased from 30–78% which is a reflection of decreased state 3 respiration rates.

When pyruvate+malate was employed as the substrate pair (table 5), the state 3 respiration rates decreased from 48–54% with T_3 treatment and from 28–30% with T_4 treatment respectively with corresponding decreases in state 4 respiration rates. Consequently, the RCR values were more or less unchanged (data not given). Thus the effects of thyrotoxicosis seemed to be different on pyruvate and malate than those seen for glutamate above (table 4). The ADP/O ratios were not altered.

Table 6. Effect of thyrotoxicosis on oxidative phosphorylation in rat heart mitochondria using succinate as the substrate.

Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		ADP-phosphorylation rate (nmol/min/mg protein)
		+ADP	−ADP	
Control (10)	1·3 ± 0·08	245·1 ± 8·5	136·0 ± 12·1	614·1 ± 37·8
0·5 μg T_3 (10)	1·1 ± 0·07	155·6 ± 3·3***	104·2 ± 5·6*	318·0 ± 22·6***
1·0 μg T_3 (10)	1·2 ± 0·10	132·1 ± 4·2***	85·8 ± 5·7**	304·5 ± 28·5***
0·5 μg T_4 (10)	1·4 ± 0·06	222·8 ± 9·8^{NS}	108·6 ± 10·5^{NS}	637·5 ± 23·4^{NS}
1·0 μg T_4 (8)	1·3 ± 0·07	156·8 ± 9·8***	78·7 ± 7·7**	398·5 ± 28·3***

Experimental details are as described in tables 1 and 4.
Results are given as mean ± SEM of the number of observations indicated in the parentheses.
*$P<0·05$, **$P<0·002$ and ***$P<0·001$ compared with control.
NS, Not significant.

Table 7. Effect of thyrotoxicosis on oxidative phosphorylation in rat heart mitochondria using ascorbate + TMPD as the substrate.

Treatment	ADP/O ratio	Respiration rate (nmol O_2/min/mg protein)		ADP-phosphorylation rate (nmol/min/mg protein)
		+ADP	−ADP	
Control (10)	0·33 ± 0·004	370·5 ± 14·6	179·6 ± 8·3	249·3 ± 9·6
0·5 μg T_3 (8)	0·32 ± 0·007	127·9 ± 7·8*	92·6 ± 3·2*	81·4 ± 5·5
1·0 μg T_3 (8)	0·33 ± 0·017	145·4 ± 9·2*	96·2 ± 8·0*	94·8 ± 6·2*
0·5 μg T_4 (10)	0·37 ± 0·050	254·6 ± 7·5*	114·8 ± 4·8*	191·3 ± 5·8*
1·0 μg T_4 (8)	0·37 ± 0·009	137·5 ± 3·3*	95·8 ± 6·7*	100·3 ± 2·9*

Experimental details are as described in tables 1 and 4.
Results are given as mean ± SEM of the number of observations indicated in the parentheses.
*$P<0·001$ compared with control.

vever, as in the case of glutamate, the ADP-phosphorylation rates decreased ificantly from 28–53%, the effects being more pronounced with T_3 treatment.

/ith succinate as the respiratory substrate, the state 3 respiration rates decreased 37–46% and 9–36% respectively with T_3 and T_4 in a dose-dependant manner. ilar pattern was also noted for the state 4 respiration rates and the extent of rease was 23–37% and 20–42% respectively (table 6).

or ascorbate + TMPD (table 7), T_3-toxicosis caused 61–66% decrease in the e 3 respiration rates with a concomitant 46–48% decrease in the state 4 iration rates. Interestingly, with T_4, both state 3 and state 4 respiration rates reased in a dose-dependant manner by 31–63% and 36–42% respectively. The eral effects of thyrotoxicosis therefore seem to be decreased state 3 respiration ch was substrate specific and thyroid hormone dose-dependant which was also ected in terms of the ADP-phosphorylation. The ADP/O ratios and respiratory trol ratios (data not given) were generally not affected by thyrotoxicosis.

'he contents of mitochondrial cytochromes as affected by thyrotoxicosis are en in table 8. It may be noted that treatment with T_3 as well as T_4 caused 29% decrease in the cytochrome aa_3 content. The contents of cytochromes

Table 8. Effect of thyrotoxicosis on the mitochondrial cytochrome contents.

	Cytochrome content, nmol/mg mitochondrial protein		
Treatment	aa_3	b	$c + c_1$
Control (10)	0·624 ± 0·024	0·384 ± 0·024	0·854 ± 0·023
0·5 μg T_3 (8)	0·442 ± 0·016***	0·345 ± 0·027*	0·758 ± 0·027**
1·0 μg T_3 (8)	0·499 ± 0·015***	0·455 ± 0·018**	0·765 ± 0·025**
0·5 μg T_4 (6)	0·448 ± 0·030***	0·390 ± 0·046^{NS}	0·907 ± 0·058^{NS}
1·0 μg T_4 (10)	0·451 ± 0·017***	0·361 ± 0·029^{NS}	0·653 ± 0·045***

Experimental details are as given in table 1 and described in the text. Contents of cytochromes were calculated from the difference spectra as described in the text. Results are given as mean ± SEM of number of observations indicated in the parentheses.

*$P < 0·05$, **$P < 0·02$ and ***$P < 0·01$ compared with control.

NS, Not significant.

T_4 (11–24% decrease). Cytochrome b content, however, increased at 1 μg T_3 dose by about 19%, while registering a decrease at a low dose of T_3 (10% decrease). Treatment with T_4 had no effect on cytochrome b content.

Discussion

These studies assume importance because in human patients thyrotoxic condition is known to cause severe cardiac dysfunctions including tachycardia, anxiety, palpitation, loss of weight and general muscular weakness (Williams and Bakke, 1982). Effect of thyrotoxicosis on mitochondrial functions have been investigated earlier by other researchers in various tissues. Some of these workers, however, have employed excessively higher doses of either T_3- or T_4- (Maley and Lardy, 1953; Hoch and Lipmann, 1954). Gustafsson *et al.* (1965) using hypothyroid rats concluded that thyroid hormones were required for maintaining the synchrony of turnover of mitochondrial protein components in the skeletal muscle. Holloszy and co-workers (Booth and Holloszy, 1975; Winder *et al.*, 1975; Winder and Holloszy, 1977) have concluded that thyrotoxicosis did not cause impairment of energy metabolism in the skeletal muscle, although turnover of cytochrome c was significantly influenced by thyroid-hormone-status. Stocker *et al.* (1968) concluded that thyrotoxicosis in dogs did not significantly influence energy metabolism in skeletal muscle mitochondria. Dow (1967) also did not find any abberation in the function of skeletal muscle mitochondria from thyrotoxic rats.

There are, not many reports in literature on the thyrotoxicosis effects on mitochondrial function of heart which comprises of specialized muscle tissue. Most of the investigators have directed their efforts to investigate effects of thyrotoxicosis on sarcolemmal and myosin ATPase activities (Goodkind *et al.*, 1974; Edelman and Ismail-Beigi, 1974; Banerjee *et al.*, 1976; Lo and Edelman, 1976; Curfman *et al.*, 1977; Litten *et al.*, 1981; Chizzonite *et al.*, 1982; Dillmann, 1982, 1985; Sinha *et al.*, 1982).

The investigations on energy metabolism reported in literature, were carried out employing either T_3- or T_4-thyrotoxicosis, at widely varying dose levels (Hoch and Lipmann, 1954; Dow, 1967; Booth and Holloszy, 1975; Winder *et al.*, 1975). In the present investigation, an attempt has been made to examine systematically the dose-dependant effects of T_3- and T_4-thyrotoxicosis. These studies assume relevance since in human patients, cases of T_3-, T_4- and combined T_3- T_4-thyrotoxicosis have been reported (Nikkila and Pitkanen, 1959; Lin and Nankin, 1980).

The present studies had to be restricted to a lower dose of the hormones since higher doses led to excessive hyperthermia, tachyacardia and increased mortality rate. Nevertheless, the present studies have brought forth the significant observations that toxicosis induced by T_3-resulted in a severe impairement of active state 3 respiration in the cardiac mitochondria than did T_4-thyrotoxicosis, with most of the substrates examined excepting ascorbate+TMPD. Interestingly the effects were substrate specific and depended on the dose of T_3 or T_4 (tables 4–7). The net result was impaired rate of ATP synthesis with all the substrates employed. The enhanced contractile response of the hyperthyroid heart referred to above and the impaired rate of ATP synthesis as observed in the present studies may explain the fatigue in the cardiac muscle leading to the congestive heart failure. Another interesting feature was, unlike in case of other tissues such as liver, kidney and brain (Hoch and Lipmann, 1954; Satav and Katyare, 1982), heart mitochondria were not uncoupled *i.e.*, their ADP/O-ratios did not decrease with any of the substrates studied. This is similar to reported observations for skeletal muscle mitochondria (Dow, 1967; Stocker *et al.*, 1968). The impairement or dysfunction therefore seems to be localized to the electron transport system rather than the phosphorylating machinery of the mitochondria.

The decreased contents of cytochromes aa_3 and $c+c_1$ can but only partly explain the decreased respiration rates. In this connection, it is interesting to note that T_3-thyrotoxicosis resulted in a generalized decrease in the contents of cytochromes aa_3 and b with a concomitant increase in the $c+c_1$ component in liver and kidney mitochondria (Winder *et al.*, 1975; Winder and Holloszy, 1977; Satav and Katyare, 1982). Impaired primary dehydrogenase functions would therefore seem to be an interesting possibility to explore. It may however be pointed out here that thyroidectomy did not result in any significant changes in the primary dehydrogenases levels in tissues such as liver, kidney and brain (Katyare *et al.*, 1977). However, Dave *et al.* (1989) have shown that the kinetic properties of succinate oxidase and ATPase change significantly in thyrotoxicosis.

Examination of T_4 and T_3 content in serum (table 2) revealed that T_3 treatment, on the one hand, suppressed serum T_4 levels while elevating the T_3 levels. T_4 treatment, on the other hand, resulted in an overall increase in the serum levels of T_3 and T_4. However, the increase in T_3 level was not of the same magnitude as in the case of T_3 treatment. These observations are in agreement with those on respiration effects, and point out to an inverse relationship between serum T_3 level and the respiratory activity. Serum T_4 levels would therefore seem to play a less significant role in influencing the respiratory activity. Interestingly, in other studies it has been observed that T_4 rather than T_3 plays a prominent role in influencing the respiratory activity in rat brain (Katyare, S. S., unpublished results). It is interesting to note in this connection that recently it has been shown that tissue levels of T_3 and T_4 are important for tissue functions (Obregon *et al.*, 1981). It is

Carter *et al.*, 1981; Satav and Katyare, 1981; Carter *et al.*, 1982; Cote' and Boulet, 1985) on tissue protein synthesis was seen since increased protein content and cardiac hypertrophy were already evident under these conditions (table 2).

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red kinetic properties of rat heart mitochondrial enzymes following rimental-thyrotoxicosis

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Abstract. Effects of T_3- and T_4-induced thyrotoxicosis on temperature-dependent Arrhenius kinetics of succinate oxidase and Mg^{2+}- and $Mg^{2+}+2,4$-dinitrophenol-dependent ATPase activities in rat heart mitochondria were examined. For succinate oxidase system, treatment with T_3 and T_4 caused increase in the energy of activation in high temperature range in a dose-dependent manner. For low temperature range, increase in energy of activation was apparent only with higher doses of the hormones; with low doses a small but reproducible decrease was evinced. The phase transition temperature decreased significantly under these conditions. For the Mg^{2+}- and $Mg^{2+}+2,4$-dintrophenol-dependent-ATPase activities, the activation energy values in high temperature range decreased in general. Activation energy values in low temperature range recorded a generalized increase in the Mg^{2+}-ATPase enzyme system while the value did not change significantly for the $Mg^{2+}+2,4$-dinitrophenol-ATPase; phase transition temperature registered a small but reproducible decrease under these conditions. The results are suggestive of increased membrane fluidization possibly through increased proportion of unsaturated fatty acids. The differential effects seen for succinate oxidase and ATPase systems are consistent with different lipid protein domains of these enzyme systems.

Keywords. Thyrotoxicosis; Arrhenius kinatics; ATPase, succinate oxidase.

duction

lation of mitochondrial membrane function by thyroid harmones is well mented (Tata *et al.*, 1963; Tata, 1964; Gustafsson *et al.*, 1965; Rajwade *et al.*, ; Satav *et al.*, 1976; Katyare *et al.*, 1977). These hormones, when administered ysiologic doses restore mitochondrial function in hypothyroid animals (Tata *et* 963; Tata, 1964; Gustafsson *et al.*, 1965), while hyperthyroidism, by contrast, to membrane dysfunction (Maley and Lardy, 1953; Hoch and Lipmann, 1954; and Katyare, 1982; Katyare and Billimoria, 1989). Studies from our atory have shown that 3, 3′, 5-triiodo-L-thyronine (T_3)- and L-thyroxine (T_4)- ced toxicosis resulted in impairment in energy coupling in heart mitochondria experimental animals (Katyare and Billimoria, 1989). Studies by other rch workers have shown that thyroid-hormone-status and hyperthyroidism lead to membrane lipid alterations (Chen and Hoch, 1976; Hoch *et al.*, 1976, ; Hoch, 1977; Gnoni *et al.*, 1980; Pasquini *et al.*, 1980; Fass and Carter, 1981,

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viations used: SDS, Sodium dodecyl sulphate; DNP, 2,4-dinitrophenol; T_t, phase transition rature; E_1, high temperature range; E_2, low temperature range; T_3, 3, 3′, 5-triiodo-L-thyronine; T_4, roxine.

1982; Hoch *et al.*, 1981; Ruggiero *et al.*, 1984). These membrane associated changes can be easily assessed in terms of possible alterations in temperature-dependent changes in enzyme kinetics (Raison *et al.*, 1971; Raison, 1972; Watson *et al.*, 1975a, b; Chen and Hoch, 1976, 1977; Hulbert, 1978). Our findings on such altered membrane characteristics of cardiac mitochondria following T_3- and T_4-thyrotoxicosis are described in the present communication. The observations reported here relate to examination of temperature-dependent changes in the activities of two enzyme systems of the respiratory assembly of heart mitochondria *viz.*, succinate oxidase and Mg^{2+}-, and Mg^{2+} + 2, 4-dinitrophenol (DNP)-dependent ATPases. The data have been analyzed in terms of Arrhenius plots (Raison *et al.*, 1971; Raison, 1972; Watson *et al.*, 1975a, b) and indicate that changes in lipid milieu may occur in the electron transport system following T_3- and T_4- induced thyrotoxicosis.

Materials and methods

Vanadium free ATP, sodium dodecyl sulphate (SDS), sodium succinate and T_3 and T_4 were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. DNP was purchased from the British Drug Houses, Dorset, Poole, UK and was recrystallized from benzene before use. Other chemicals used were of analytical-reagent grade.

Treatment of animals with T_3 and T_4 and isolation of heart mitochondria was according to the procedures already described (Katyare and Billimoria, 1989).

Succinate oxidase activity

Succinate oxidase activity was measured with a Clark-type oxygen electrode in a medium (total volume 1·3 ml) containing 67 mM potassium phosphate buffer pH 7·4, 0·4 mM $CaCl_2$ and 0·4 mM $AlCl_3$ essentially as described by Katyare *et al.* (1971). Mitochondria were incubated in the reaction medium for 2–5 min for maximum activation of the enzyme (Thorn, 1962; Katyare *et al.*, 1971) and respiration was then initiated with addition of 10 mM sodium succinate.

ATPase activity

The Mg^{2+}-, and Mg^{2+} + DNP-dependent ATPase activities (Pullman and Penefsky, 1963) were measured in a medium (1 ml) containing: 50 mM Tris-acetate buffer, pH 7·4, 2 mM $MgCl_2$ and 50–180 μg of mitochondrial proteins; 0·1 mM DNP was included wherever indicated. After incubation for 2–5 min, the reaction was started by adding 2 mM ATP (adjusted to pH 7·4 with Tris base). The reaction was terminated at the end of 10 min with 0·1 ml of 10% (w/v) SDS (Shallom and Katyare, 1985) and the inorganic phosphate liberated was estimated according to the method of Fiske and Subba Row (1925).

Linearity of the reaction with time under the assay condition was ascertained in separate experiments (data not given).

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

The analytical treatment of the data was essentially as described (Raison *et al.*, 1971; Raison, 1972; Watson *et al.*, 1975a, b). The log of specific activity of the were determined.

Results

Figure 1 represents the temperature-dependent changes in succinate oxidase activity in heart mitochondria as influenced by T_3- and T_4-induced thyrotoxicosis. It can be noted that the maximum activation of the succinate oxidase activity occurred between 42–46°C. Nevertheless, the extent of activation was different for the various experimental groups compared to the control. It may be seen that the Arrhenius plots (figure 2) indeed reflect the changes induced by thyrotoxicosis with respect to the energies of activation in the high and low temperature ranges and T_t. The representative values for energies of activation and T_t are shown on the plots and mean values calculated from replicate observations are given in table 1. Thus it can be noted that energies of activation on the high temperature range (E_1) increased progressively with increasing doses of T_3 and T_4. Similar pattern was also noted for the energy of activation at low temperature range (E_2) particularly at the higher doses of T_3 and T_4. This agrees well with earlier observations on decreased succinate oxidation rates (Katyare and Billimoria, 1989). The T_t also showed progressive decrease with increasing doses of T_3 and T_4 and in fact at higher dose of the hormones, the T_t had decreased by almost 20°C.

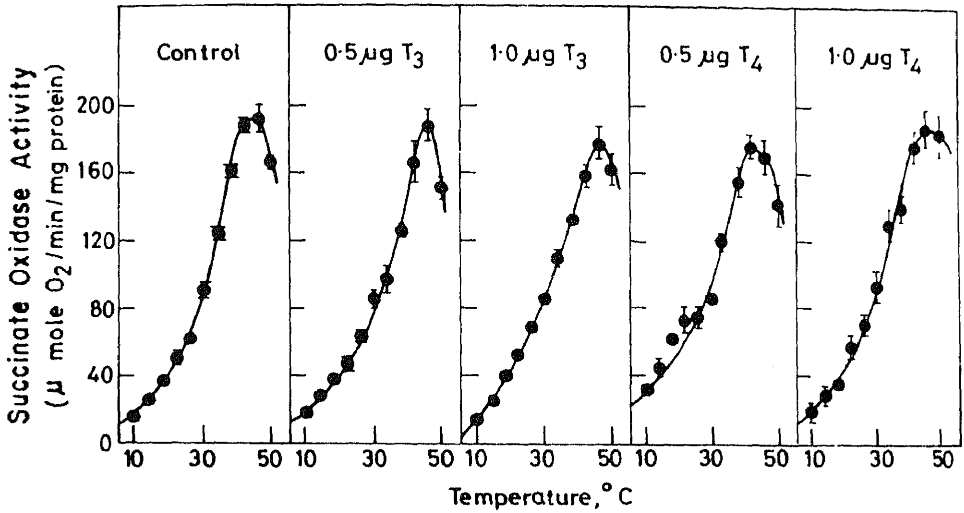

Figure 1. Temperature-dependent changes in succinate oxidase activity in rat heart mitochondria following T_3 and T_4 treatment. Male rats were given either T_3 or T_4 (0·5 or 1 µg hormone/g body weight) for 4 days. Succinate oxidase activity was measured with a Clark-type oxygen electrode.

Results are given as mean ± SEM of 4 animals.

The results on Mg^{2+}-ATPase activities as influenced by T_3- and T_4- treatment are shown in figure 3. The differential effect of thyrotoxicosis is once again apparent

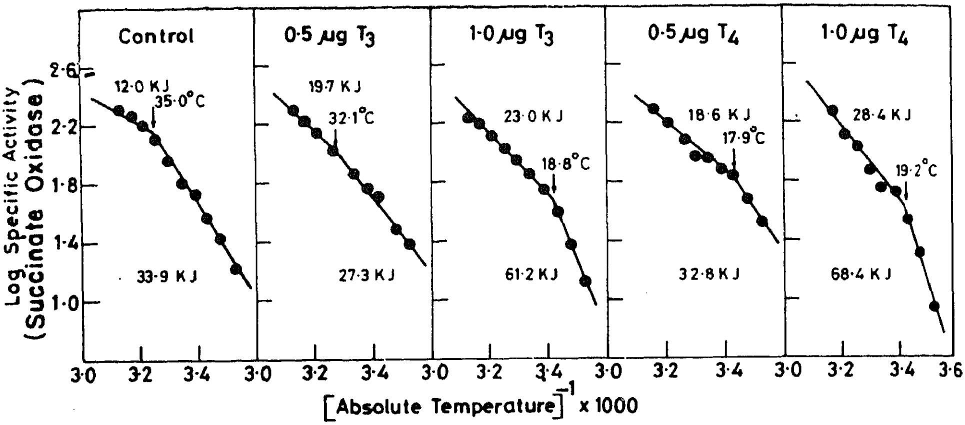

Figure 2. Arrhenius plots of log succinate oxidase activity against reciprocal of absolute temperature in rat heart mitochondira following T_3 and T_4 treatment.

The experimental details are as given in figure 1. Results are typical of 4 independent animals. Calculation of E_1, E_2 and T_t were as detailed in the text.

Table 1. Effect of thyrotoxicosis on succinate oxidase activity in rat heart mitochondria

Treatment	Energy of activation (KJ/mol) E_1	Energy of activation (KJ/mol) E_2	T_t
Control	9·1 ± 1·74	34·1 ± 1·42	38·0 ± 0·16
0·5 µg T_3	20·2 ± 1·04*****	29·5 ± 0·77*	34·4 ± 1·25*
1·0 µg T_3	24·6 ± 0·55*****	55·8 ± 4·95***	18·7 ± 0·34*****
0·5 µg T_4	18·9 ± 1·21***	27·9 ± 1·30**	20·4 ± 0·51*****
1·0 µg T_4	26·2 ± 2·81****	57·1 ± 5·13***	19·4 ± 1·40*****

Animals were injected with doses of T_3 or T_4 as indicated/g body weight for 4 consecutive days.

Other experimental details are as described in the text.

Results are given as mean ± SEM of 4 independent experiments.

$*P<0{\cdot}05$, $**P<0{\cdot}02$, $***P<0{\cdot}01$, $****P<0{\cdot}002$ and $*****P<0{\cdot}001$ compared with control.

and is also reflected in Arrhenius plots shown in figure 4. The observed changes relating to energies of activation and T_t are summarized in table 2. Thus, it can be noted that in contrast to the succinate oxidase system, the values of E_1 decreased particularly at higher doses of T_3 and T_4 while the E_2 values increased in general except for 1 µg T_4 group. The value of T_t also decreased but to a lesser extent except for the 1 µg T_4 group, where no change in T_t was evident.

The data for Mg^{2+} + DNP-ATPase activities are shown in figures 5 and 6 respectively, and the values of E_1, E_2 and T_t are summarized in table 3. It can be noted that once again as in the case of Mg^{2+}-ATPase activity the values of E_1 decreased particularly very significantly at the higher doses of the hormones while those for E_2 showed a generalized increase. Decrease in T_t values was also recorded but this was of lesser magnitude, compared to the succinate oxidase system.

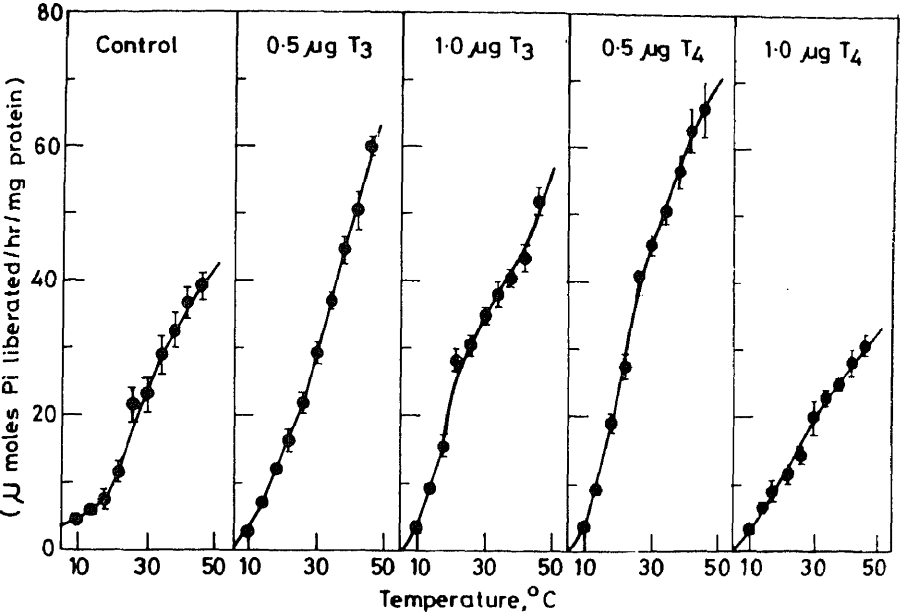

Figure 3. Temperature-dependent changes in Mg^{2+}-ATPase activity in rat heart mitochondria following T_3 and T_4 treatment.

Results are given as mean ± SEM of 4 independent animals.

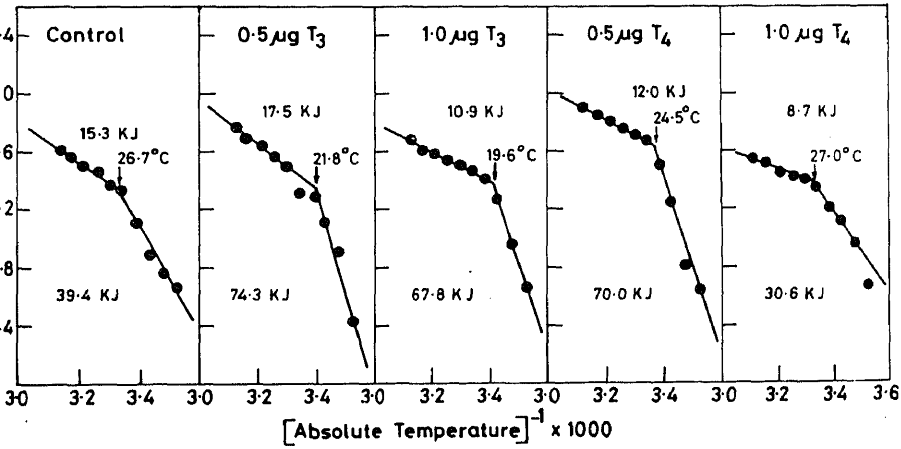

Figure 4. Arrhenius plots of log Mg^{2+}-ATPase activity against reciprocal of absolute temperature in rat heart mitochondria following T_3 and T_4 treatment.

Results are typical of 4 independent animals.

ussion

present investigations were undertaken to examine lipid-dependent perturba-
s in the membrane functions of rat heart mitochondria following treatment with
or T_4. The results have clearly shown that the energies of activation for
inate oxidase increased in general, following hormone treatment whereas the T_t

Table 2. Effect of thyrotoxicosis on Mg^{2+}-ATPase activity in rat heart mitochondria.

	Energy of activation (KJ/mol)		
Treatment	E_1	E_2	T_t
Control	15·6 ± 1·44	37·7 ± 2·73	28·5 ± 1·08
0·5 μg T_3	19·1 ± 1·30^{NS}	63·7 ± 6·78**	24·1 ± 1·05*
1·0 μg T_3	11·3 ± 0·70*	69·2 ± 10·51*	21·6 ± 0·009****
0·5 μg T_4	13·1 ± 0·77^{NS}	68·3 ± 3·23***	23·3 ± 0·40***
1·0 μg T_4	9·8 ± 0·45**	30·9 ± 1·13^{NS}	28·7 ± 1·06^{NS}

Experimental details are as given in table 1.

Results are given as mean ± SEM of 4 independent experiments.

*$P<0·05$, **$P<0·01$ and ***$P<0·002$ and ****$P<0·001$ compared with control.

NS, Not significant.

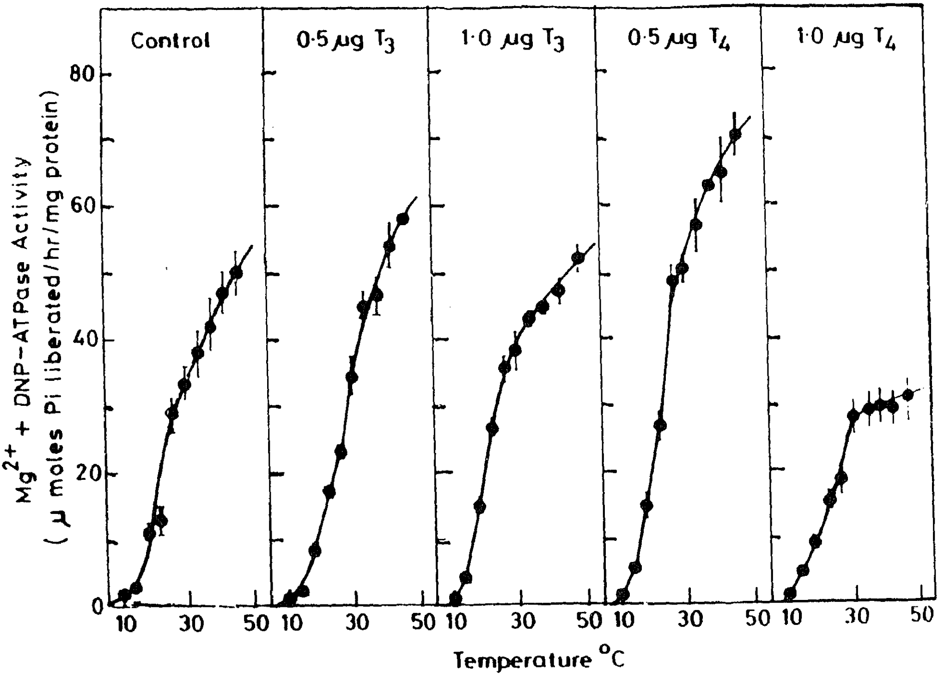

Figure 5. Temperature-dependent changes in Mg^{2+} + DNP-ATPase activity in rat heart mitochondria following T_3 and T_4 treatment.

Results are given as mean ± SEM of 4 independent animals.

decreased significantly (almost 20°C decrease). For the Mg^{2+}- and Mg^{2+} + DNP-dependent ATPase, the picture is somewhat different, *i.e.*, the energies of activation E_1 decreased in general following hormone treatment except in case of 0·5 μg T_3 and T_4 treatment. The values of E_2 increased only for the Mg^{2+}-ATPase activity. The T_t recorded consistently a small but reproducible decrease amounting to 5–7°C. The observed changes are therefore indicative of differential alterations in the lipid environments of the two enzyme systems. Decrease in the T_t is taken as indicative of the increased degree of unsaturation in the fatty acids (Raison *et al.*,

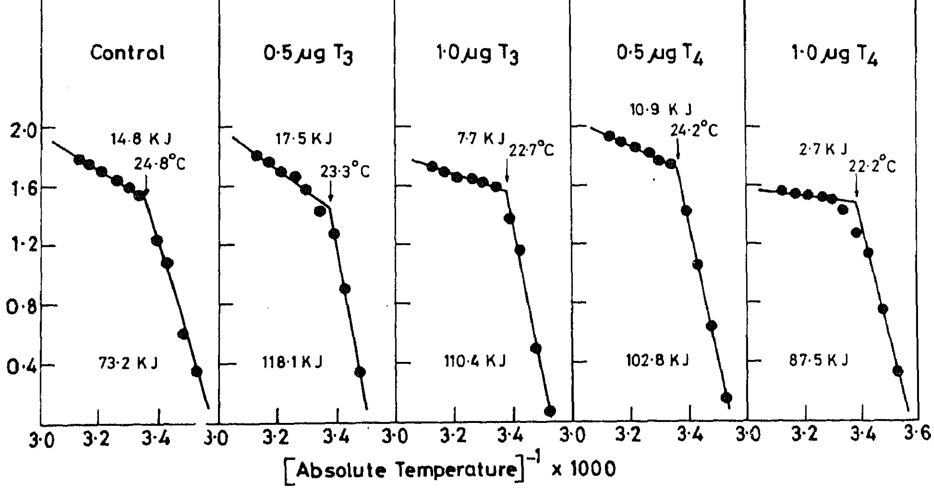

Figure 6. Arrhenius plots of log Mg^{2+} + DNP-ATPase activity against reciprocal of absolute temperature in rat heart mitochondria following T_3 and T_4 treatment.

Results are typical of 4 independent animals.

Table 3. Effect of thyrotoxicosis on Mg^{2+} + DNP-ATPase activity in rat heart mitochondria.

Treatment	Energy of activation (KJ/mol)		
	E_1	E_2	T_t
Control	15·0 ± 1·13	88·8 ± 10·19	27·1 ± 0·58
0·5 μg T_3	18·0 ± 0·55*	117·3 ± 9·44^{NS}	22·7 ± 0·44*
1·0 μg T_3	7·2 ± 0·26***	116·7 ± 12·55^{NS}	21·9 ± 0·62*
0·5 μg T_4	9·0 ± 1·13**	92·9 ± 3·06^{NS}	24·2 ± 0·56*
1·0 μg T_4	2·1 ± 0·34***	103·1 ± 12·53^{NS}	22·1 ± 0·38*

The experimental details are as given in table 1 and in the text.
Results are given as mean ± SEM of 4 independent experiments.
$*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with control.
NS, Not significant.

71; Raison, 1972; Watson *et al.*, 1975a, b). In this connection, it is interesting to te that in hypothyrodism total unsaturation of phospholipid fatty acid in rat liver tochondrial inner membrane decreased significantly by 10% (Chen and Hoch, 76). Abnormal Arrhenius profile for adenine nucleotide carrier of rat liver tochondria in hypothyroid rats has been reported which was corrected after a gle injection of L-T_4 (Hoch, 1977). The polyunsaturated fatty acid contents of the ıer mitochondrial membrane were also similarly corrected (Chen and Hoch, 1976; ɔch *et al.*, 1976). The desaturation of essential polyenoic fatty acids is also celerated by thyroid hormones *in vitro* (Fass *et al.*, 1972). Thyroid-hormone-pendent changes in fatty acid composition of microsomal and mitochondrial ids in rat liver have also been reported by other research workers (Fass and rter, 1981, 1982; Hoch *et al.*, 1981; Ruggiero *et al.*, 1984).

It is of interest to note that the effects were differential for the succinate oxidase and the ATPase systems. The succinate oxidase spans almost completely the entire respiratory chain and therefore several components of the electron transport system are involved. It would be obvious that the temperature-dependent changes observed for succinate oxidase system represent a composite and complex process involving several respiratory chain components. By contrast, for the ATPase system, the lipid-protein interactions pertain only to the single enzyme domain.

It may be mentioned here that the specific dependency of mitochondria ATPase on linoleoyl cardiolipin has been shown (Lopez-Moratalla *et al.*, 1973). Lipid-dependency of other mitochondrial enzymes such as β-hydroxybutyrate dehydrogenase, succinate dehydrogenase and cytochrome oxidase has also been reported (Vik and Capaldi, 1977; Abuirmeileh and Elsen, 1980; Ashraf *et al.*, 1980; Fry and Green, 1980, 1981). Stimulation of fatty acid biosynthesis in the liver of thyrotoxic rat and decreased phospholipid content and choline and CDP-choline incorporation in phosphatidyl choline in mitochondrial fraction have been reported (Gnoni *et al.*, 1980; Pasquini *et al.*, 1980). It is possible that the observed changes in profiles of succinate oxidase and ATPase systems may correlate with the observed decrease in state 3 respiration rates and ADP-phosphorylation rates (Katyare and Billimoria, 1989).

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tease inhibitors from jackfruit seed (*Artocarpus integrifolia*)

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Abstract. Protease inhibitory activity in jackfruit seed (*Artocarpus integrifolia*) could be separated into 5 fractions by chromatography on DEAE-cellulose at pH 7·6. A minor fraction (I) that did not bind to the matrix, had antitryptic, antichymotryptic and antielastase activity in the ratio 24:1·9:1·0. Fraction II bound least tightly to the ion exchanger eluting with 0·05 M NaCl and could be resolved into an elastase/chymotrypsin inhibitor and a chymotrypsin/trypsin inhibitor by chromatography on either immobilized trypsin or phenyl Sepharose CL-4B. Fractions III and IV eluted successively with 0·10 M NaCl and 0·15 M NaCl from DEAE-cellulose, inhibited elastase, chymotrypsin and trypsin in the ratio 1·0:0·53:0·55 and 1·0:8·9:9·8 respectively. Fraction V, most strongly bound to the matrix eluting with 0·3 M NaCl and was a trypsin/chymotrypsin inhibitor accounting for 74% of total antitryptic activity. This inhibitor was purified further. The inhibitor with a molecular weight of 26 kd was found to be a glycoprotein. Galactose, glucose, mannose, fucose, xylose, glucosamine and uronic acid were identified as constitutent units of the inhibitor. Dansylation and electrophoresis in the presence of mercaptoethanol indicated that the inhibitor is made up of more than one polypeptide chain. The inhibitor combined with bovine trypsin and bovine α-chymotrypsin in a stoichiometric manner as indicated by gel chromatography. It had very poor action on subtilisin BPN′, porcine elastase, pronase, *Streptomyces caespitosus* protease and *Aspergillus oryzae* protease. It powerfully inhibited the caseinolytic activities of rabbit and horse pancreatic preparations and was least effective on human and pig pancreatic extracts. Modification of amino groups, guanido groups and sulphydryl groups of the inhibitor resulted in loss of inhibitory activity. Reduction of disulphide bridges, reduction with sodium borohydride and periodate oxidation also decreased the inhibitory activity.

Keywords. Jackfruit seed; protease inhibitors; isolation; glycoprotein.

duction

tree (*Artocarpus integrifolia* Family: *Moraceae*) is grown widely in the torial regions for its large edible summer fruits. The over-size seeds from this leguminous plant is also edible even though it is believed that it is difficult to igested (Siddappa, 1957). Powerful trypsin inhibitory activity was reported in seed and it was shown that the activity could be destroyed by boiling the eous solution of the seed powder at 100°C for 30 min (Siddappa, 1957). Studies this laboratory indicated the presence of chymotrypsin and trypsin inhibitors is seed and it was also shown that cooking the whole seed for 30 min at 100°C not destroy the inhibitory principles (Bhat and Pattabiraman, 1985). Jackfruit extract was found to inhibit the proteolytic activities of different animal creatic preparations effectively (Bhat and Pattabiraman, 1986). In this

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eviations used: BAPNA, Benzoyl-DL-arginine *p*-nitroanilide; ATEE, N-acetyl-L-tyrosine ethyl ester; bovine serum albumin; PHMB, *p*-hydroxymercuribenzoate; TNBS, trinitrobenzene sulphonate; , O-methylisourea; EA, ethylacetamidate; CHD, 1, 2-cyclohexane dione; DTNB, 5, 5′-dithiobis-2- benzoic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; M_r, cular weight.

communication, we report the presence of multiple forms of protease inhibitors in jackfruit seed which differ in their action on trypsin, chymotrypsin and elastase and in heat stability. The different forms however, appear to belong to the Kunitz family of inhibitors, based on their molecular size. The isolation and characterization of the major inhibitor are also reported.

Materials and methods

Materials

Jackfruit seeds were collected from ripe fruits locally, sun-dried and stored at −5°C. Bovine trypsin (twice crystallized), bovine α-chymotrypsin (thrice crystallized), Kunitz soybean trypsin inhibitor, pepsin, subtilisin BPN′, pronase, *Aspergillus oryzae* protease (type XXIII), *Streptomyces caespitosus* protease (type VI), acetone powders of rabbit, horse, rat, dog, pig and ox pancreas, benzoyl DL-arginine *p*-nitroanilide (BAPNA), N-acetyl-L-tyrosine ethyl ester (ATEE), cytochrome c, myoglobin, bovine serum albumin (BSA), lysozyme, carbonic anhydrase, trypsinogen, bovine enterokinase, ovalbumin, *p*-hydroxy-mercuribenzoate (PHMB) and sodium trinitrobenzene sulphonate (TNBS) were the products of Sigma Chemical Company, St. Louis, Missouri, USA. Porcine elastase (twice crystallized) was obtained from Millipore Biochemical Corporation, Freehold, USA. O-Methylisourea (OMI), ethylacetamidate (EA) and 1,2-cyclohexane dione (CHD) were obtained from Aldrich Chemical Company, Milwaukee, USA. Ninhydrin, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 2-mercaptoethanol, bicinchoninic acid protein assay reagent and Coomassie brilliant blue protein assay reagent were purchased from Pierce Chemical Company, Rockford, USA. Sodium borohydride was the product of Polysciences Inc., Warrington, USA. Affigel-10 was the product of Bio-Rad Laboratories, Richmond, USA. Phenyl Sepharose CL-4B and Sephadex G-50 and G-100, were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Human pancreatic acetone powder was prepared in the laboratory from the tissues obtained during autopsy. Pancreatic acetone powders were activated using bovine enterokinase as described previously (Prabhu *et al.*, 1984). Other reagents were commercial samples of analytical grade.

Methods

Preparation of the seed extract: Thirty g of jackfruit seed powder was homogenized with 300 ml of 0·1 M phosphate buffer (pH 7·6), stirred for 20 min and centrifuged at 12,000 *g* for 20 min at 4°C. To the supernatant (185 ml), 72·2 g of ammonium sulphate was added. After 18 h standing at 4°C, the precipitate was collected by centrifugation at 12,000 *g* for 20 min, dissolved in 0·02 M phosphate buffer (pH 7·6) and dialyzed against 5 litres of the same buffer for 18 h. This fraction (60 ml) was used for further studies.

The ammonium sulphate fraction was applied to a column of DEAE-cellulose (bed vol. 30 ml, 1·6 × 15·0 cm^2) equilibrated with 0·02 M phosphate buffer (pH 7·6). The column was washed with 150 ml of the equilibration buffer. The column was eluted in 4 stages with 0·02 M phosphate buffer containing 0·05 M NaCl, 0·1 M NaCl, 0·15 M NaCl and 0·3 M NaCl (each system, 150 ml). Fifteen ml actions were collected at a flow rate of 30 ml h^{-1} and analyzed. The results are iown in figure 1. The ratios of trypsin : chymotrypsin : elastase inhibitions of the fferent fractions were as follows: II, 0·24 : 0·67 : 1·0; III, 0·55 : 0·53 : 1·0; IV, 8 : 8·9 : 1·0; V, 220 : 206 : 1·0.

hromatography on Sephadex G-50: The 4 inhibitor fractions (II–V) separated on EAE-cellulose were subjected to gel chromatography as follows. Aliquots of action II (13 mg protein), fraction III (15 mg), fraction IV (8 mg) and fraction V 9 mg) were applied to columns of Sephadex G-50 (bed vol. 40 ml, 0·9 × 65 cm^2) uilibrated with 0·02 M phosphate buffer (pH 7·6) containing 0·1 M NaCl. The lumn was eluted with the equilibration buffer. Two ml fractions were collected at flow rate of 8 ml h^{-1} and analyzed for protease inhibitor activities.

iromatography on phenyl Sepharose CL-4B: Aliquots of inhibitor fractions otained by DEAE-cellulose chromatography were applied to phenyl Sepharose lumns (bed vol. 8 ml, 0·9 × 13 cm^2) equilibrated with 0·02 M phosphate buffer ntaining 1·5 M NaCl. The column was eluted successively with 100 ml of the uilibration buffer, 100 ml of the buffer containing 0·5 M NaCl and finally with 0 ml of the buffer. Eight ml fractions were collected at a flow rate of 10 ml h^{-1} d analyzed. Fraction II (20·5 mg protein), fraction III (30 mg protein), fraction IV mg protein) and fraction V (19 mg protein) from DEAE-cellulose chromatography re used in these studies.

iromatography on trypsin-Affigel 10 and chymotrypsin-Affigel 10: Trypsin-Affigel d chymotrypsin-Affigel were prepared as described elsewhere (Udupa and ttabiraman, 1985). The inhibitor fractions from DEAE-cellulose were applied to lumns of immobilized trypsin or immobilized chymotrypsin (bed vol. 6 ml, × 10 cm^2) equilibrated with 0·02 M phosphate buffer (pH 7·6) containing M NaCl. The column was washed with 30 ml of the equilibration buffer and ited with 30 ml of 50 mM HCl. Six ml fractions were collected at a flow rate of nl h^{-1} and analyzed. Aliquots of fraction II (15 mg protein), fraction III (12·5 mg otein, fraction IV (5 mg protein) and fraction V (19 mg protein) obtained from EAE-cellulose chromatography were used in these studies.

olation of the major trypsin/chymotrypsin inhibitor: Fraction V separated by EAE-cellulose chromatography (156 mg protein, 3·24 × 10^4 trypsin inhibitor units) s applied to a column of chymotrypsin-Affigel 10, equilibrated with 0·02 M osphate buffer (pH 7·6) containing 0·3 M NaCl and washed with 50 ml of the ne buffer. The bound inhibitor was eluted with 50 mM HCl (flow rate 8 ml h^{-1} 4°C, ten 10 ml fractions). Fraction No. 7 was dialyzed against 0·02 M phosphate, 7·6 and used for further studies.

say of protease inhibitory activity: Caseinolytic activities of proteases and their iibition were assayed as described before (Udupa and Pattabiraman, 1987). Ten of trypsin, 9 μg of α-chymotrypsin, 15 μg of elastase, 44 μg of pronase, 100 μg of *caespitosus* protease, 660 μg of *A. oryzae* protease, 10 μg of subtilisin BPN′, 3 μg (protein) of human, 50 μg of bovine, 90 μg of pig, 112 μg of rabbit, 156 μg of , 120 μg of dog and 96 μg of horse pancreatic preparations were used in the einolytic assay. Amidolytic activity of trypsin (15 μg) was assayed using BAPNA substrate and its inhibition was studied as described before (Prabhu *et al.*, 1984). e inhibition of the amidolytic activities of human (182 μg protein), bovine

(203 μg), pig (136 μg), rabbit (224 μg), rat (310 μg) and horse (150 μg) pancreatic preparations was also studied similarly. Esterolytic activity of chymotrypsin (0·9 μg) using ATEE as substrate and its inhibition were studied as detailed elsewhere (Prabhu *et al.*, 1984). The inhibition of the esterolytic activities of human (51 μg protein), bovine (7·2 μg), pig (18 μg), rabbit (45 μg), rat (64 μg) and horse (35 μg) pancreatic preparations were also studied similarly. Unless stated otherwise all inhibition studies were done by the caseinolytic method. One unit of inhibitory activity by the caseinolytic method is equivalent to the amount that suppressed the production of trichloroacetic acid soluble fragments corresponding to one optical density unit (540 nm) under the assay conditions. The units were calculated based on the linear range of inhibition.

Electrophoresis: Polyacrylamide gel (7·4%) electrophoresis (PAGE) was performed using 0·04 M Tris-glycine buffer, pH 8·3 at a current of 4 mA/tube for 4 h. Electrophoresis in the presence of 0·1% sodium dodecyl sulphate (SDS) in the presence and in absence of 1% mercaptoethanol was done for 10 h at 8 mA/tube at pH 7·2 using 10% gel. Protein bands were stained with Coomassie brilliant blue R-250. For molecular weight (M_r) determination, lysozyme, Kunitz soybean trypsin inhibitor, carbonic anhydrase, ovalbumin and BSA were used as standards. About 100 μg protein of the purified inhibitor was used in these studies.

Gel chromatography: The purified inhibitor (600 μg protein) was subjected to gel chromatography on Sephadex G-100 (bed vol. 36 ml, $0{\cdot}9 \times 60$ cm^2, equilibration buffer 0·02 M phosphate, pH 7·6 containing 0·1 M NaCl). One ml fractions were collected at a flow rate of 6 ml h^{-1} and the fractions were analyzed for protein, antitryptic and antichymotryptic activities. Cytochrome c, myoglobin, trypsinogen, pepsin and BSA were used as markers for M_r determination.

For complex formation studies, 600 μg (protein) of the inhibitor was mixed with 500 μg of active bovine trypsin or 500 μg of active bovine chymotrypsin in 1 ml of 0·02 M phosphate buffer (pH 7·6) containing 0·1 M NaCl. The solution was subjected to chromatography as described above. Trypsin and chymotrypsin were also subjected to gel chromatography under similar conditions.

Carbohydrate analysis: Carbohydrate content of the purified inhibitor was determined by the phenol-sulphuric acid reaction (Du Bois *et al.*, 1956) using D-glucose as standard. The individual monosaccharides were quantitated as follows. The inhibitor (24 mg protein) was hydrolyzed with 8 ml of 2 N H_2SO_4 in sealed tubes at 105°C for 10 h. The hydrolyzate was neutralized with barium carbonate. The suspension was centrifuged and the supernatant was collected. The precipitate was washed 4 times with water (20 ml). The supernatant and washings were pooled and flash evaporated under vacuum at 55–60°C. The concentrate was separated into neutral, acidic and basic sugar fractions by chromatography on coupled columns of Dowex-50, and Dowex-1 as described by Spirol (1960). The concentrate was applied to Dowex-50, H^+ (bed vol. 2·5 ml) and the effluent was passed through Dowex-1, $HCOO^-$ (bed vol. 2·5 ml). The washing containing the neutral monosaccharides was lyophilized and subjected to descending paper chromatography on Whatman No. 1 paper for 84 h using n-butanol : ethanol : water (10 : 1 : 2 v/v) as solvent system. The spots were revealed by spraying with ammoniacal $AgNO_3$. Regions corresponding to the spots in an unstained paper were eluted with water and the individual monosaccharides were quantitated by the od of Nelson (1944) with appropriate standards. Hexosamine bound to Dowex- was eluted with 20 ml of 2 N HCl. The eluate was evaporated to dryness under uum, flushed twice with water and the residue dissolved in water was used for osamine estimation (Levvy and McAllan, 1959) with D-glucosamine. HCl as dard. The Dowex-1 column was eluted with 40 ml of 0·5 M formic acid. The te was evaporated to dryness and uronic acid was estimated (Bitter and Muir) using D-glucuronic acid as standard.

he presence of N-glycosidic link in the inhibitor was tested as per the method of and Scocca (1972). An aliquot of the inhibitor solution (3 mg carbohydrate valent) was treated with 1 M $NaBH_4$ in 1 M NaOH (vol. 4·5 ml) for 6 h at 98°C ealed tubes. The solution was acidified to pH 5 with glacial CH_3COOH and lted on a column of Bio-Gel P-2. The carbohydrate fractions from the column pooled and hydrolyzed with 6 N HCl at 100°C for 6 h. The solution was orated to dryness and subjected to descending paper chromatography on tman No. 1 paper for 24 h using pyridine:ethyl acetate:water:acetic acid :3:1) as solvent system. Glucosaminitol and glucosamine spots were revealed praying with ammoniacal $AgNO_3$. Glucosaminitol and glucosamine moved 31 35 cm respectively from the origin.

no acid analysis: The inhibitor sample was hydrolyzed with 6 N HCl in sealed uated tubes at 110°C for 24 h and samples were analyzed in an amino acid yzer. Tryptophan in intact protein was determined spectrophotometrically by method of Gaitonde and Dovey (1970). The inhibitor protein was dansylated as ribed by Boulton and Bush (1964) for 10 h at 30°C. The sample was hydrolyzed 6 N HCl at 105°C for 24 h in sealed tubes. The hydrolyzate was flash orated under vacuum at 60°C. The residue was extracted with aceton:water) and subjected to thin layer chromatography on silica gel G using r:methanol:acetic acid (100:50:1) as solvent. The spots were visualized under light.

nical modification studies: The effect of modification of guanido groups by ydrin (Chaplin, 1976) and CHD (Abe *et al.*, 1978) was studied as described re (Udupa and Pattabiraman, 1985). Modification of amino groups by TNBS nes *et al.*, 1967), EA (Hunter and Ludwig, 1972) and OMI (Kimmel 1967) was described earlier (Udupa and Pattabiraman, 1985). Modification of SH groups HMB (Benesch and Benesch, 1962) and DTNB (Ellman, 1959) and the effect on itory activities were also studied. Periodate oxidation was performed as ws. The inhibitor (200 μg protein) in 0·02 M acetate buffer (pH 5) was treated 0·02 M $NaIO_4$ at 4°C, in a volume of 2 ml. At definite intervals, aliquots were drawn, dialyzed against 0·02 M phosphate buffer (pH 7·6) and assayed for ual inhibitory activities. To determine the effect of $NaBH_4$ 200 μg of the itor protein was treated with 0·2 M $NaBH_4$ in 0·02 M phosphate (pH 7·6). uots were withdrawn at intervals, dialyzed against 0·02 M phosphate (pH 7·6) assayed for residual protease inhibitory activities.

ein assay: For routine purposes, protein was assayed by the method of Lowry (1951), using BSA as standard. Protein content of the purified inhibitor was assayed by the bicinchoninic acid method (Smith *et al.*, 1985) and by the dye ng method (Bradford, 1976).

Phosphate buffer extract of jackfruit seeds showed inhibition of trypsin, chymotrypsin and elastase in the ratio 6·7:6·5:1·0. When the extract was subjected to heat treatment at 100°C for 10 min, there was negligible loss of antitryptic and antichymotryptic activities, whereas antielastase activity was reduced by 50%. When the extract was stored at 4°C, there was a gradual loss of antielastase activity (per cent loss after 5, 9 and 15 days 16·5, 33·3 and 41·7%). The decrease in antitryptic and antichymotryptic activities after 15 days storage was less than 5%. The data suggest that jackfruit seed may contain more than one inhibitor and the inhibitor contributing to action against elastase is relatively labile.

When the crude extract (ammonium sulphate fraction) was subjected to chromatography on DEAE-cellulose, small amount of inhibitory activity was found in the break-through fluid. This fraction accounting for 0·83% of total antitryptic activity, inhibited trypsin, chymotrypsin and elastase in the ratio 2·4:1·9:1·0. Because of its rapid loss of activity on standing and due to its low quantity, this fraction was not studied further. Bulk of the activity bound to DEAE-cellulose, could be eluted into 4 fractions using a discontinuous gradient elution. The data are shown in figure 1. The most anionic fraction (V) accounted for the bulk of the antitryptic and antichymotryptic activity. It had negligible antielastase action. Fraction IV (0·15 M NaCl eluant) had comparable antitryptic and antichymotryptic activities, but it showed more antielastase activity than fraction V. Fraction III (0·10 M NaCl eluant) and fraction II (0·05 M NaCl eluant) were more active on elastase. On rechromatography on DEAE-cellulose, the individual fractions were eluted in the same region as in the initial chromatography indicating that they are not experimental artefacts.

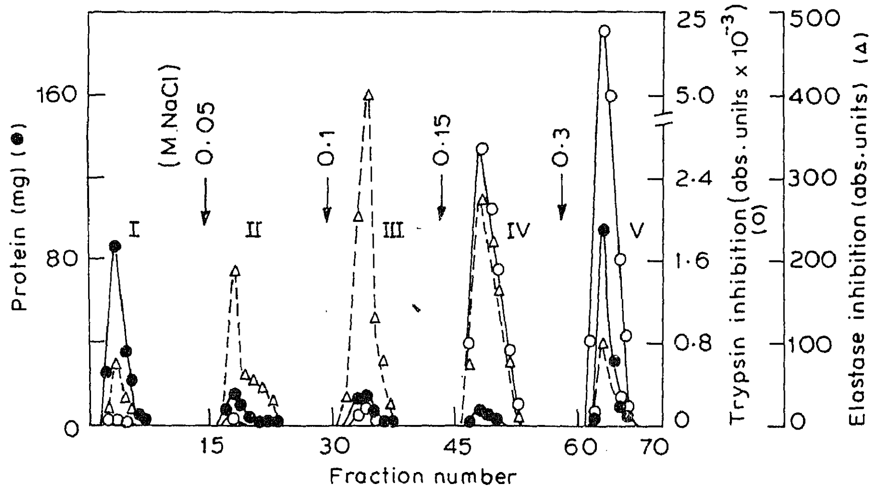

Figure 1. Discontinuous gradient elution of protease inhibitory activities from a DEAE-cellulose column. Experimental details are described under methods. For the sake of clarity antichymotryptic activities are not presented.

The 4 inhibitor fractions were eluted with similar V_e values on gel chromatography phadex G-50. The V_e/V_o values suggest that the 4 inhibitors have M_r in the 20–25 kd. Fraction V was found to be heat stable. No loss of antitryptic or ıymotryptic activity was noticed on heat treatment for 5 min at 100°C. Nearly of the inhibitory activities were lost with fractions IV and III under similar tions. Fraction II was completely heat labile.

e inhibitor fractions were subjected to affinity chromatography on trypsin-l and chymotrypsin-Affigel. Fraction V was bound to both the immobilized ıes and was eluted with 50 mM HCl with good recoveries (trypsin-Affigel: yptic activity 90%, antichymotryptic activity 90%, chymotrypsin-Affigel: 98 99%). Similarly, fraction IV was bound and was eluted with acid, with eries of antitryptic, antichymotryptic and antielastase activities of 80, 88 and on trypsin-Affigel and 80, 92 and 67% on chymotrypsin-Affigel. Fraction III behaved similarly. However, fraction II displayed a different pattern. During n-Affigel chromatography it separated into two fractions. All the antielastase ty (recovery 80%) and some amount of antichymotryptic activity (16%) were ered in the buffer wash. This fraction had no antitryptic activity. The acid contained the trypsin inhibitory activity (recovery 70%) and the remaining otrypsin inhibitory activity (61%). On the other hand, during chromatography ymotrypsin-Affigel all the 3 activities were bound to the matrix and eluted 50 mM HCl with good recovery (80–85%). It can be surmised that fraction II ts of an elastase/chymotrypsin inhibitor and a trypsin/chymotrypsin inhibitor. s is supported by hydrophobic chromatography on phenyl Sepharose. When on II was subjected to chromatography, the break-through volume (0·02 M hate containing 1·5 M NaCl) contained antitryptic and antichymotryptic y. The eluant (buffer containing 0·5 M NaCl) had antielastase and anti-otryptic activity but negligible antitryptic activity. On the other hand, ons III, IV and V were eluted from phenyl Sepharose as single peaks. ons III and IV were eluted with buffer containing 0·5 M NaCl. Fraction V l more tightly to the column was eluted with 0·02 M buffer.

ction V from DEAE-Cellulose that accounted for the major portion of the yptic and antichymotryptic activities was further purified by chromatography ymotrypsin-Affigel. The data on purification are summarized in table 1.

. Purification of the major trypsin/chymotrypsin inhibitor from jackfruit seeds.

	Total protein (mg)	Antitryptic activity			Antitryptic/antichymotryptic activity ratio
		Inhibitor units	Specific activity	Per cent recovery	
extract	2200	100,000	45·3	100	1·13
nium sulphate fraction	924	69,000	74·7	69	1·12
-cellulose fraction	156	32,400	208	32·4	1·12
trypsin-Affigel fraction	85	28,000	329	28·0	1·10

PAGE, the inhibitor moved as a single protein band (figure 2A). On SDS-E without mercaptoethanol, a single band profile was again obtained

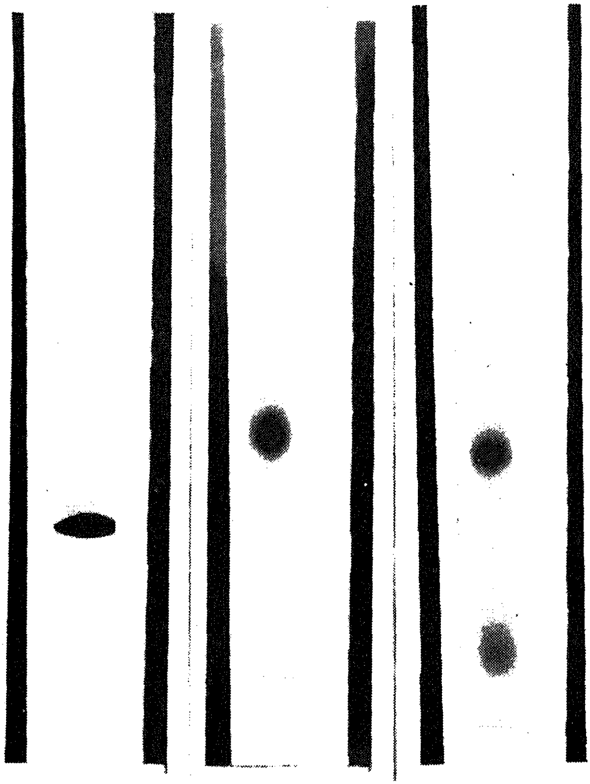

Figure 2. Electrophoresis of the purified inhibitor. Lane 1, PAGE; Lane 2, SDS-PAG Lane 3, SDS-PAGE with mercaptoethanol. Experimental details are given under metho

contaminant protein. The M_r of the native inhibitor by SDS-PAGE was estimat as 26·6 kd (figure 3A). The two major protein fragments in the presence of t reducing agent were calculated to have M_r value of 26 and 14 kd. It can concluded that the inhibitor is a dimer linked by disulphide bridges. This supported by N-terminal analysis. Two dansyl derivatives corresponding to vali and leucine were identified on processing the purified inhibitor. In addition, a fai spot corresponding to the glycine derivative was seen. On gel chromatography Sephadex G-100, the inhibitor was eluted as a single peak with constant rates antitryptic and antichymotryptic activity across the peak (figure 3B). The M_r of t inhibitor based on gel chromatographic data was found to be 26 kd (figure 3B inse The amino acid composition of the inhibitor is shown in table 2. For compariso the composition of 3 Kunitz type inhibitors are shown. The purified jackfruit se inhibitor was poor in methionine, histidine and tryptophan like the other inhibito

The inhibitor was found to be a glycoprotein. Based on phenol-sulphuric ac reaction, the purified inhibitor was found to have 70·8 μg carbohydrate (as gluco per mg protein. The neutral sugars identified in the acid hydrolyzate of the inhibit were galactose, glucose, mannose, xylose and fucose (figure 4) in the mol proportion of 1·0 : 1·3 : 2·0 : 1·8 : 1·40. In addition, hexosamine and uronic acid we identified and were present in the molar proportion of 3·25 : 3·22. The inhibitor

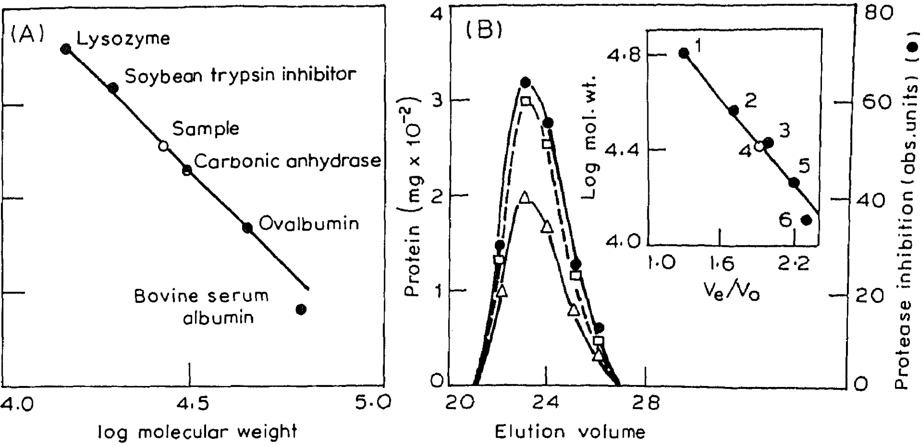

Figure 3. (A) M_r determination by SDS-PAGE. (B) Chromatographic profile of the inhibitor on Sephadex G-100. (●), Trypsin inhibition; (○), chymotrypsin inhibition; (△), protein. *Inset*: M_r determination by gel chromatography. (1), Cytochrome C (12·4 kd); (2), myoglobin (17 kd); (3), trypsinogen (24 kd); (4), inhibitor; (5), pepsin (34·7 kd); (6), BSA (66 kd). Experimental details are given under methods.

Table 2. Amino acid composition (mol per cent) of inhibitors.

Amino acid	Jackfruit seed*	Brazilian Carolina seed	Kunitz soybean	Winged bean
Asp	13·20	12·40	15·50	10·70
Thr	4·88	3·45	4·17	4·94
Ser	10·60	6·90	6·55	9·40
Glu	10·80	19·30	10·70	12·50
Gly	10·30	11·30	9·52	12·50
Ala	4·29	11·00	4·76	6·36
Val	7·03	2·75	8·33	2·35
Met	—	—	1·19	—
Ileu	5·45	6·21	9·60	5·93
Leu	11·60	10·70	8·93	10·90
Tyr	7·04	4·82	2·30	5·50
Phe	6·71	4·82	5·30	4·20
His	2·52	—	1·10	0·67
Lys	5·31	6·20	5·95	6·42
Trp	0·5	0·68	1·19	1·10

*Proline, half-cystine and arginine were not determined. For other seeds, values were calculated from published data; Brazilian Carolina (Richardson *et al.*, 1986), winged bean (Shibata *et al.*, 1986), Kunitz soybean (Koide *et al.*, 1973).

ce of glucosaminitol suggesting the presence of N-glycosidic link involving amine in the inhibitor.

purified inhibitor was fairly heat stable. Per cent loss of antitryptic activity at treatment at 100°C for 15, 30 and 60 min was 47, 73 and 87%. sponding values for antichymotryptic activity were 38, 56 and 69%. The ely more stable nature of antichymotryptic activity was confirmed by heat ent studies at lower temperatures. At 90°C, after 15 and 30 min, the loss in

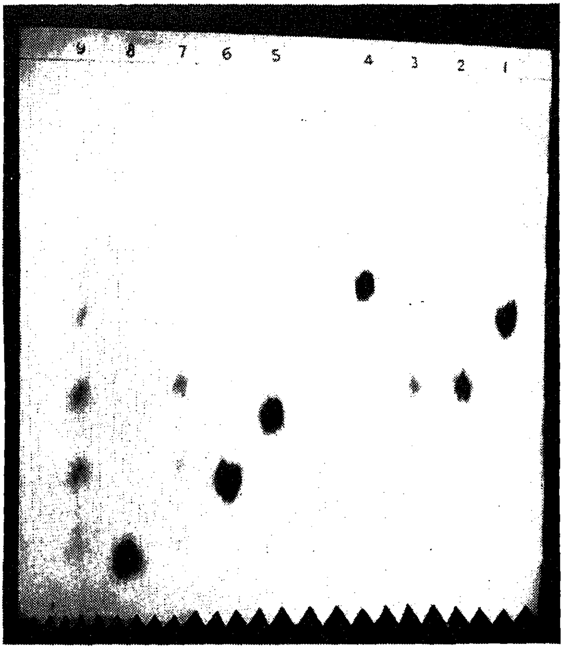

Figure 4. Paper chromatography of neutral monosaccharide units from the inhibitor. (1), Glucose; (2), fructose; (3), mannose; (4), galactose; (5), arabinose; (6), xylose; (7, 9), inhibitor hydrolyzate; (8), fucose. Experimental details are given under methods.

antitryptic activity was 20 and 50% whereas the loss in antichymotryptic activity was 0 and 8%. At 80°C for 60 min, 50% of the antitryptic activity was lost whereas no decrease in antichymotryptic activity was noticed. The inhibitor was stable on exposure to pH values in the range 1–9 for 12 h at 4°C. There was a gradual loss of activity in the more alkaline range. At pH 10, about 10% of antitryptic activity and 7·5% of antichymotryptic activity were lost. At pH 12, the residual antitryptic and antichymotryptic activities were 35 and 32%. The inhibitor did not lose its activities on treatment with equal weights (protein) of pepsin (at pH 2), pronase (at pH 7) and elastase (at pH 7) for time duration up to 12 h. When equimolar amounts of inhibitor and trypsin or chymotrypsin were incubated at pH 3·3 at 37°C for 4 h, there was no decrease in antitryptic or antichymotryptic activity when measured at pH 7·6.

Based on inhibition in the linear range, by the caseinolytic method, it was observed that one mol of inhibitor inactivated 2·75 mol of bovine trypsin and 2·60 mol of bovine α-chymotrypsin. Using BAPNA as substrate, the mol ratio of interaction between trypsin and the inhibitor was found to be 2·44 : 1·0. To test whether the high ratios are due to underestimation of protein levels of the inhibitor by the Lowry's method, protein was determined by other methods. By the bicinchoninic acid assay, the protein value was 97·6% of that determined by the method of Lowry *et al.* (1951). By the dye binding method, the protein value was low (26% compared to Lowry's method). The data suggest that the high molar ratio for interaction of the inhibitor and trypsin (or chymotrypsin) is not due to underestimation of protein and could be due to the nonspecific inactivation of the

:s induced by the inhibitor during the assay conditions. However, when was used as substrate for chymotryptic action, the ratios of interaction, n the inhibitor and the enzyme was calculated to be 1·0: 1·09.

nplex formation studies by gel chromatography indicated stoichiometric tion between the inhibitor and the enzymes (figure 5). The calculated M_r of

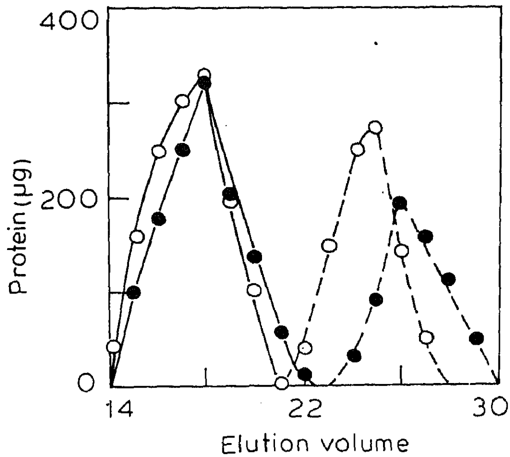

Figure 5. Studies on complex formation on Sephadex G-100. (–●–), Trypsin + inhibitor; (–○–), chymotrypsin + inhibitor; (—●—), trypsin alone; (—○—), chymotrypsin alone. Experimental details are given under methods.

or: trypsin complex and inhibitor: chymotrypsin complex were 43 kd, lower e estimated value. The trypsin: inhibitor complex had no antitryptic activity nt (10% of native inhibitor) antichymotryptic activity (data not shown). ly, the chymotrypsin: inhibitor complex had no antichymotryptic activity d 18·5% of antitryptic activity compared to equivalent amount of native or (data not shown). The data suggest that one protease can partially replace er from the complex. The presence of mutually exclusive binding sites for and chymotrypsin were ruled out by mixed enzymes assay. When lytic inhibition of trypsin (5 µg), chymotrypsin (5 µg) and a mixture of the zymes (2·5 µg each) by the inhibitor (1 µg) was studied under the routine conditions, the magnitudes of inhibition were 56·7, 51·7 and 53·2% vely for the 3 systems.

modes of inhibition of trypsin and chymotrypsin by the purified inhibitor nd to be uncompetitive (figure 6). Based on Lineweaver-Burk plots, the K_i calculated with reference to trypsin and chymotrypsin were $1{\cdot}29 \times 10^{-8}$ M 22×10^{-8} M respectively. The inhibitor had very poor action on other ytic enzymes by the caseinolytic method. The relative extent of inhibition of elastase, subtilisin BPN′, pronase, *A. oryzae* protease and *S. caespitosus* e were 0·6, 2·4, 1·2, 1·6 and 1·7% compared to trypsin (100%). The effect of

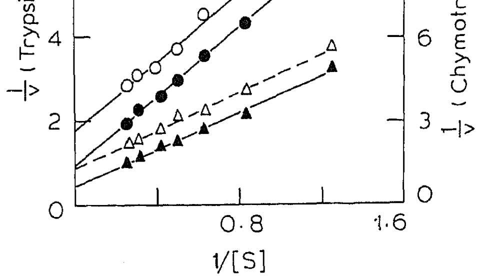

Figure 6. Mode of inhibition of trypsin and chymotrypsin by the purified inhibitor. (●), trypsin; (○), trypsin + inhibitor (6 μg); (▲), chymotrypsin; (△), chymotrypsin + inhibitor (6 μg).

Table 3. Effect of the inhibitor on different pancreatic preparations.

Per cent inhibition	Rabbit	Horse	Bovine	Rat	Dog	Human	Pig
μg inhibitor required to reduce caseinolytic activity							
25	0·70	0·75	0·94	0·94	1·00	1·60	1·88
50	1·42	1·50	1·90	1·90	2·00	3·20	4·63
75	2·55	2·30	5·40	5·00	5·50	7·50	20·0
μg inhibitor required to reduce BAPNA hydrolysis							
25	1·17	0·75	1·56	0·93	1·00	2·68	1·90
50	2·35	1·50	3·10	1·87	2·00	5·40	3·80
75	4·30	2·30	5·80	4·80	3·10	9·00	6·20
μg inhibitor required to reduce ATEE hydrolysis							
25	0·27	0·80	0·20	0·30	0·30	0·60	0·45
50	0·55	1·60	0·38	0·80	0·60	1·20	0·90
75	1·55	3·20	0·60	3·20	1·53	2·50	1·90

systems. With reference to tryptic inhibition, dog and horse enzymes were most susceptible and human and pig enzymes, least susceptible. Rabbit and bovine chymotrypsins were found to be powerfully inhibited compared to the human and horse enzymes.

The results on chemical modification of the inhibitor and the effect on inhibitory activities are shown in table 4. The loss of activity on treatment with guanido group modifiers (CHD and ninhydrin) and amino group modifiers (TNBS, OMI and EA) was slow with respect to the time of interaction. However, when the inhibitor was subjected to heat treatment at 80°C for 5 min, prior to treatment with ninhydrin or OMI, there was relatively rapid loss of activity, suggesting that the functional groups in native inhibitor are not easily available for modification. PHMB and DTNB also inactivated the inhibitor implicating the SH groups in the expression of

Table 4. Effect of chemical modification on the action of the inhibitor.

		Per cent residual activity against	
Modifier	Time (h)	Trypsin	Chymotrypsin
Ninhydrin	24	50	56
	48	40	50
Ninhydrin[a]	6	20	60
	24	0	0
CHD	24	56	65
	48	50	63
TNBS	24	75	85
	48	70	70
EA	24	68	71
	48	48	57
OMI	24	70	66
	48	63	45
OMI[a]	2	50	60
	24	20	15
	48	0	0
PHMB	24	56	71
	48	0	0
DTNB	24	62	66
	48	0	0
Mercaptoethanol	24	75	61
	48	68	56
$NaIO_4$	24	65	56
	48	60	54
$NaBH_4$	2	33	30
	6	5	5
	12	0	0

[a]Inhibitor solution heat treated at 80°C for 5 min prior to modification.

tivity. Treatment with mercaptoethanol also caused a gradual decrease in activity, dicating that S–S bridges are essential for the functional integrity of the inhibitor. alO_4 also caused a decrease in inhibitory activity. Similarly, $NaBH_4$ treatment used a reduction in inhibitory activity culminating in almost complete loss in 6 h.

iscussion

ckfruit seed, is shown to contain at least 6 distinct inhibitors. Five of them could -separated based on their charge characteristics by chromatography on DEAE-llulose. One fraction separated on DEAE-cellulose, can be further separated into chymotrypsin/elastase inhibitor and a trypsin/chymotrypsin inhibitor by romatography on immobilized trypsin. These two could also be separated by

1979, 1980). The isoinhibitors from Brazilian Carolina seed and winged bean have been separated by ion-exchange chromatography. Two of the winged bean inhibitors had strong action on trypsin and 4 others were essentially chymotrypsin inhibitors (Shibata *et al.*, 1986). Three inhibitors from okra seed were shown to be trypsin/chymotrypsin inhibitors and the fourth one, an exclusive trypsin inhibitor (Ogata *et al.*, 1986). The erythrina seed inhibitors have been classified into trypsin/chymotrypsin/plasminogen activator inhibitors, trypsin inhibitors and chymotrypsin inhibitors (Joubert *et al.*, 1987). Among the 6 silk tree inhibitors, 3 are trypsin/chymotrypsin inhibitors and remaining chymotrypsin/elastase inhibitors. Based on the present study, it can be concluded that jackfruit seed contains two trypsin/chymotrypsin inhibitors, an elastase/chymotrypsin inhibitor and 3 trypsin/chymotrypsin/elastase inhibitors. In all probability, the different inhibitors are the outcome of limited substitution of amino acid residues. This is supported by the sequence studies with isoinhibitors of Brazilian Carolina seed and the amino acid composition of erythrina seeds. It is known that substitution of Arg-63 by Trp in Kunitz soybean trypsin inhibitor makes it a powerful chymotrypsin inhibitor (Kowalski *et al.*, 1974).

The major trypsin/chymotrypsin inhibitor from jackfruit seed characterized in this study can be classified as a Kunitz type of inhibitor based on the molecular size and amino acid composition. However, this inhibitor has some interesting features. It appears to be made up of more than one polypeptide chain linked by disulphide bridges. Secondly, the inhibitor is shown to be a glycoprotein. In this context it is pertinent to point out that all the 8 isoinhibitors of Brazilian Carolina seed are composed of a large α-chain and a smaller β-chain linked by disulphide bonds (Richardson *et al.*, 1986). In addition, the acacia and albizzia protease inhibitors are also known to be made up of more than one polypeptide chain linked by disulphide bridges (Kortt and Jermyn, 1981; Odani *et al.*, 1980). Even though the presence of carbohydrates in many plant protease inhibitors have been indicated by phenol-sulphuric acid reaction (Ogata *et al.*, 1986), conclusive evidence for the presence of oligosaccharide units linked to peptide by N-glycosidic link has been provided only with the inhibitor from Barbados pride seeds (Hase *et al.*, 1986). This inhibitor also belongs to the Kunitz family.

The relative inaccessibility of the major jack bean inhibitor is indicated by slow rate of interaction of amino group and guanido group modifiers. Brief heat treatment of the inhibitor prior to addition of modifiers enhanced the rate of disappearance of the inhibitory activity suggesting the exposure of the target sites. A similar observation has been made with a trypsin/chymotrypsin inhibitor from the millet, echinocloa (Udupa and Pattabiraman, 1985). Even though periodate oxidation and reduction with $NaBH_4$ resulted in decrease/loss of inhibitory activity, it does not provide conclusive evidence for the role of carbohydrates in the functional integrity of the inhibitor. It is well known, periodate can attack side chain residues of polypeptides. It has been shown that peptide bonds can be reduced to a limited extent by sodium borohydride in many proteins (Paz *et al.*, 1970).

While complex formation studies indicated stoichiometric interaction between the inhibitor and trypsin or chymotrypsin, calculation of molar ratio of interaction between the inhibitor and enzymes from the extent of decrease of proteolytic activity in the conventional assay, provided much higher values. The uncompetitive mode of inhibition observed during kinetic analysis of inhibition suggests that the itude of inhibition increases with increase in substrate concentrations. Oste (1987) observed that many furans and pyrroles inhibited carboxypeptidase A neir effects can be characterized as substrate induced inhibition. It is probable he protease inhibitor from jackfruit seed behaves in a similar fashion.

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Protein deficiency and age related alterations in rat peritoneal macrophage lipids

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Abstract. The effects of dietary protein restriction and age on the thioglycollate elicited peritoneal macrophage lipid constituents were studied. Impact of subtle changes in lipid components on macrophage functions have been assessed. Lipid profiles of macrophages recovered from rats fed 20 and 4% protein diets and stock diet fed rats (0 and 3 wk) were comparable qualitatively. Quantitative analysis however revealed significant decrease in phospholipids (30–40%) and consequent elevation of cholesterol/phospholipid molar ratios in the protein depleted and young rats (0 wk), compared to the protein fed groups. The protein deficient and the young rats also exhibited accumulation of certain neutral lipids and reduction in triglycerides. Analysis of fatty acid methyl esters of macrophage phospholipids revealed the predominance of long chain polyunsaturated fatty acids even when oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) formed the bulk of unsaturated fatty acids in the diet. However, the long chain poly unsaturated fatty acid content, particularly the docosahexaenoic acid ($C_{22:6n\text{-}3}$) was greatly reduced in the protein depleted and 0 wk rats. Observed changes in the long chain polyunsaturated fatty acids of macrophage phospholipids may be of physiological significance as they modulate the immunological functions of the cell.

Keywords. Protein deficiency; peritoneal macrophages; lipids; cholesterol/phospholipid ratio; fatty acid composition.

Introduction

Macrophages rank among the most important cells which guarantee the optimum course of immune reactions. Recently much attention has been paid to the role of dietary fat in immune response (Smith *et al.*, 1985). Macrophage mediated immune functions such as phagocytosis and cytotoxicity are regulated by critical composition of the cell membrane glycolipids (Schlager *et al.*, 1983). Protein restriction has adverse effects on various aspects of immune competence (Salimonu *et al.*, 1982). Though proteins and lipids are essential components of cell membranes, many of the membrane functions have been traditionally associated with proteins. However, there is sufficient evidence to suggest that lipids also greatly influence the cellular functions (Spector and Yorek, 1985). In macrophages the membrane associated endocytic functions apart from being receptor mediated, are known to be governed by the fluidity of the membranes (Mahoney *et al.*, 1980). The physical state of the membrane lipids can modulate the physiological function of the membranes. Various reports have suggested a role for lipids and their functions (De La Fuente, 1985; Smith *et al.*, 1985). However, these effects are not well elucidated. The present studies were carried out to characterise the changes in the macrophage lipid profile, particularly in their fatty acyl chains in response to protein deficiency and underdevelopment.

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Abbreviations used: PBS, Phosphate buffered saline; FAME, fatty acid methyl esters; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Materials and methods

Animals and diet

Male weanling Wistar rats weighing 40–50 g were fed stock diet (table 1) *ad libitum* and on reaching about 100 g body weight, were separated randomly into 4 groups of 10. One group of animals was injected intraperitoneally with 5 ml of 3% thioglycollate (Difco product). Three days later, they were sacrificed by anesthetizing with ether and macrophages were isolated. Remaining 3 groups of animals were each fed *ad libitum* isocaloric stock, 20 and 4% protein diets (table 1) respectively. The low protein diet was nutritionally adequate except for protein levels. Each animal was housed separately in a hanging type cage and its food intake and body weight were recorded. Animals had continuous access to food and water in a temperature and light controlled room (23–25°C, 12 h light-dark cycle). At the end of 3rd week the animals were sacrificed on the 3rd day following thioglycollate injection.

Table 1. Composition of diets.

Stock diet		Experimental diet (% protein)		
Ingredient	Amount (%)	Ingredient	20 amount %	4 amount %
Cracked wheat	70	Casein	20	4
Cracked Bengal gram	20	VSD	2	2
Fish meal	5	Salt mixture	4	4
Yeast powder	4	Sugar	9	9
Shark liver oil	1	Seasame oil (til oil)	6	6
		Corn starch	59	75

The experimental diets were designed to be isocaloric to stock diet which provided about 350–360 cal/100 g of diet.

100 g of VSD (vitaminised starch dextrose) comprised of all water soluble vitamins totalling up to 12·14 g, 37·86 g sugar and 50 g starch. Seasame (til) oil (6 g) was fortified to contain 2000 IU, vitamin A palmitate, 1000 IU ergocalciferol and 5 mg dl-α-tocopherol acetate.

Isolation of macrophage

Peritoneal macrophages were isolated (Edelson *et al.*, 1975) using cold 0.1 M phosphate buffered saline (PBS), pH 7.2. The collected peritoneal fluid was rifuged at 600 *g* for 10 min at 4°C. Peritoneal cells were freed of contaminating ırocytes with hypotonic NH_4Cl (0·85%). The resultant pellet was transferred sterile plastic petridishes (9·5 cm diameter) and suspended in Eagles minimum ıtial medium (Difco product), containing 100 μg/ml each of penicillin and otomycin and incubated for 2 h at 37°C. The nonadherent cells were removed vashing 4–5 times with saline. The adhering cells were scrapped with a rubber eman and suspended in saline. A giemsa stained preparation of this suspension observed under light microscope and 95% of these cells were identified as ophages, almost free of lymphocytes and other cells. Cells were counted at 10X a hemocytometer.

tion of macrophage membrane

rophage membrane was isolated by using Tris-HCl buffer pH 7·7 essentially as ibed by Snyderman *et al.* (1984). To assess the membrane purity, 5′-eotidase activity was estimated using adenosine monophosphate as the rate (Widnell and Unkeless, 1968).

analysis

cells were made 0·1% with Triton X-100 and homogenised and proteins were ated according to the method of Miller (1959). The cells from 5 rats were ed and the lipids were extracted with chloroform:methanol 2:1 (v/v) as ibed by Folch *et al.* (1957). The extracts were pooled, evaporated under N_2 total lipids estimated colorimetrically (Bragden, 1951). Lipids were separated thin-layer chromatography (TLC) using hexane:diethylether:acetic acid 15:1 (v/v)]. Lipid containing regions were visualized by exposure to I_2 vapours the separated fractions were scrapped and eluted with chloroform and ated by specific colorimetric methods. Membrane lipids were also similarly cted and analysed. Cholesterol and cholesterol esters were estimated by the edure outlined by Sperry and Webb (1950). Triglycerides were estimated using motrophic acid reagent (Lambert and Neish, 1950). Fatty acids were estimated escribed by Duncombe (1963). Phospholipids were estimated (Charles and art, 1980) using lysophosphatidyl ethanolamine as the standard.

acid analysis and gas chromatography

phospholipids were eluted and fatty acid methyl esters (FAME) were ıred by transesterification (Mahoney *et al.*, 1977). Phospholipid samples ıg) were heated with 300–400 μl of 6% HCl in methanol and heated for 16 h at under N_2. After evaporating excess methanolic HCl, the residue was dissolved small volume of water (0·5 ml) and methyl esters were extracted with equal ne of distilled hexane thrice. The pooled extracts were evaporated and solved in hexane and analysed. The stock diet fat (obtained by Soxhlet ction) and seasame oil (400 μg each) used in the prepared diets were directly esterified and their total FAME were analysed.

increased at 4°C/min, after initial 4 min hold up to the final temperature of 210°C till the end of the analysis (40 min). The temperature of the injection port was 230°C. The results are reported as per cent composition of each FAME compared with the total identified fatty acid methyl esters. The following FAME standards obtained from Sigma Chemical Co., St. Louis, Missouri, USA, in order of their elution were examined. $9{:}0$, $12{:}0$, $14{:}0$, $16{:}0$, $16{:}1_{n-7}$, $18{:}0$, $18{:}1_{n-9}$, $18{:}2_{n-6}$, $20{:}0$, $18{:}3_{n-3}$, $20{:}1_{n-9}$, $20{:}3_{n-9}$, $22{:}0$, $20{:}4_{n-6}$, $22{:}1_{n-9}$, $20{:}5_{n-3}$, $24{:}0$, $24{:}1$, $22{:}5_{n-3}$ and $22{:}6_{n-3}$. The position of the double bond is given when known with the omega classification.

Results and discussion

Growth profile of animals

The rats fed 20% diet gained weight (both total body wt. and wt./g of diet), in a linear pattern during the experimental period. The immediate physiological response due to protein restriction reflected in a reduction in weight which remained constant, till the end of the experiment. Their body weights were comparable to those of the young rats used in the 0 wk control group. The food intake and weight gain pattern of the stock diet fed, 3 wk control rats was similar to those of the 20% protein fed rats.

Macrophage cell count

Macrophages recoverable from the 20% protein and stock diet fed rats, possessing a normal linear growth pattern in response to thioglycollate stimulation was higher. The total cell count per rat was reduced by about 62% in the 4% protein fed rats compared to the 20% protein fed animals (figure 1). The young rats (0 wk control) had 41% lower cells than the 3 wk control rats. Protein per 10^6 cells was comparable in all the 4 groups. However the total lipid per 10^6 cells was nearly 2-fold in the protein deficient and young animals compared to the other 2 groups. Consequently lipid to protein ratio was about 1 in the 20% protein and 3 wk stock diet fed rats. It increased to 2·4 in the 4% protein and 0 wk stock diet fed groups.

Macrophage lipid profile

Starting from $1{\cdot}222 \times 10^8$ cells, a membrane preparation exhibiting 3·2-fold purification compared to the crude homogenate, as indicated by 5′-nucleotidase activity was obtained. The recovery in terms of protein was only 22·7% though in terms of enzyme activity it was 72·8%. However the yield of membrane fraction in terms of protein was only 7·9 μg/10^6 cells. In the initial standardisation experiments it was observed that the TLC profiles of lipid fraction of the whole macrophages and isolated membrane were identical. The percentage distribution (table 2) of individual lipids were comparable. Slight decrease in cholesterol and cholesterol ester and increase in phospholipids in the membrane compared to whole cells,

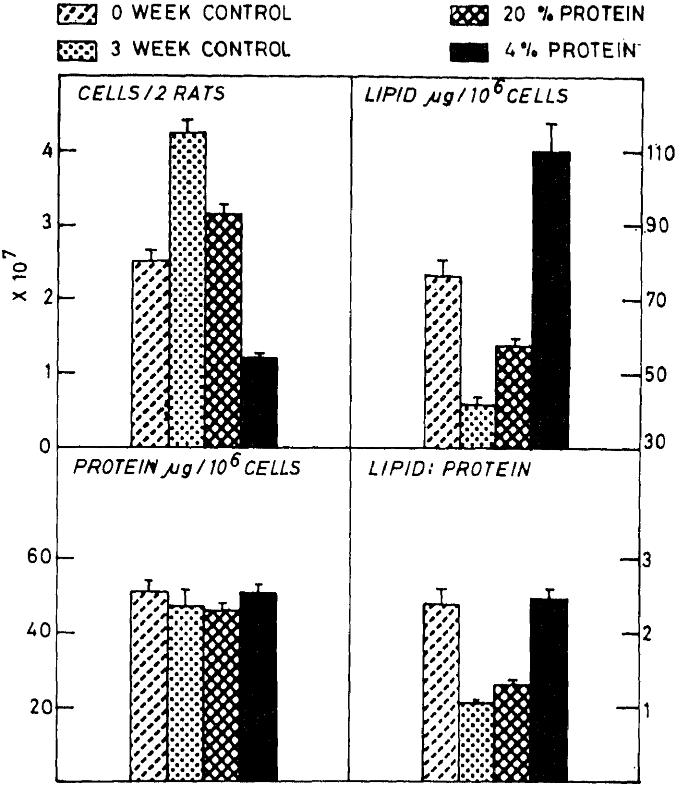

Figure 1. Cell count and composition of thioglycollate elicited macrophages. The cells were isolated from the peritoneal cavity by cold PBS washing and incubated in sterile petridishes. The non adherent cells were washed away and the adherent macrophages recovered and counted. The cellular protein and lipid contents were estimated.

Table 2. Comparison between lipid composition of macrophage whole cells and membrane.

Lipid component	Distribution of lipids (%)	
	Macrophage membrane	Macrophage cells
Cholesterol	8·28	10·76
Cholesterol ester	2·17	3·16
Phospholipid	48·5	43·25
Triglycerides	6·2	7·9
Free fatty acids	10·28	11·75
Other lipids	24·56	23·18
Lipid/protein ratio	1·92	0·6
Cholesterol/phospholipid	0·45	0·67

Values are mean of duplicate experiments. Ten rats were used in each set. The lipids were extracted, separated and quantitated as detailed in the text.

reflected in decreased cholesterol/phospholipid molar ratio of the membrane. The membrane preparation required large number of cells. However, the differences were very little in absolute terms. Hence in the subsequent studies dealing with experimental animals, where the results were comparative, whole macrophages were directly used for lipid extraction.

The profile of lipids (figure 2) was identical in all the groups, though they varied quantitatively. Phospholipids were reduced by 30–40% in the protein restricted and 0 wk groups compared to the other two groups. Cholesterol was slightly lower in the 4% group which was compensated by the corresponding increase in the cholesterol ester fraction (table 3). Consequently the cholesterol/phospholipid molar ratio was 0·67 and 0·63 in the 3 wk and 20% groups while it was about 1·0 in the other two groups. This increase would alter membrane fluidity and permeability factors that govern functional properties of macrophages.

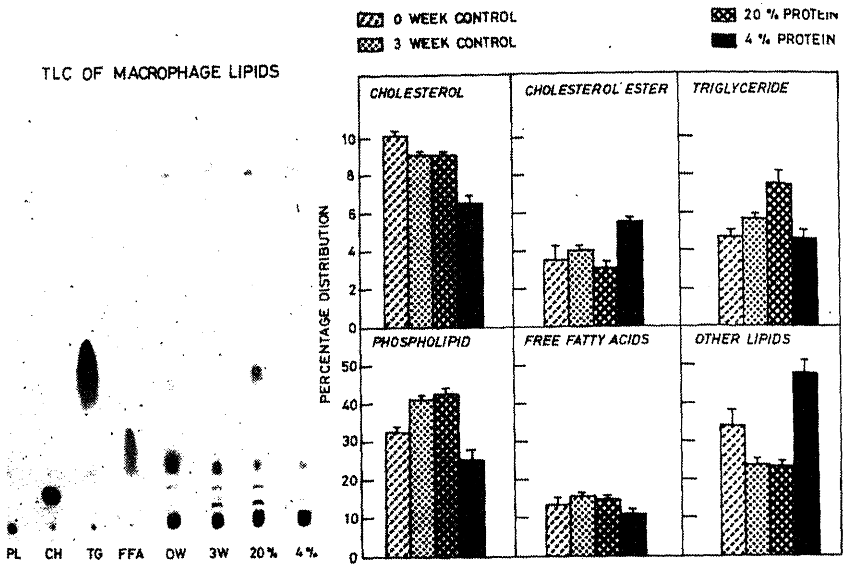

Figure 2. Distribution of macrophage lipid components. Lipids separated by TLC were visualized by exposure to iodine vapours. The lipid spots were scrapped and then eluted with chloroform. The components were estimated by specific colorimetric methods. Values represent mean ± SD of 8 independent estimations and are expressed as per cent of total.

Table 3. Macrophage cholesterol and phospholipid content.

Groups	Cholesterol	Cholesterol ester	Total cholesterol	Phospholipid	Cholesterol: phospholipid molar ratio
	(μg/mg protein)				
0 wk control	23·9 ± 2·4	8·44 ± 2·26	32·35 ± 4·53	76·13 ± 13·46	0·91 ± 0·04
					$P \leqslant 0.0001$
3 wk control	30·09 ± 3·62	12·94 ± 1·34	43·03 ± 4·74	134·49 ± 16·66	0·67 ± 0·01
20% protein	30·09 ± 2·99	11·25 ± 2·0	41·34 ± 4·96	137·57 ± 18·16	0·63 ± 0·03
					$P \leqslant 0.0001$
4% protein	16·87 ± 1·10	17·15 ± 6·07	34·02 ± 7·03	67·23 ± 15·15	1·07 ± 0·03

The results are expressed as mean ± SD of 8 independent estimations. The contents are expressed as μg/mg of total macrophage protein.

Triglycerides were more in the protein fed groups but free fatty acids did not differ significantly. The other lipid fractions comprising of the mono and diglycerides and unidentified lipids increased by about 40–100% in the young and protein restricted groups. Schmidt *et al.* (1984) have shown active turnover of phospholipids in macrophages during their activation. The synthesis of these lipid components are enzymatically mediated. Significant protein deficiency induced changes in rat liver membrane lipids have been reported earlier (Gerson, 1974).

Macrophage phospholipid fatty acids

The FAME analysis of the dietary fat (figure 3) indicated that $C_{18:1}$ and $C_{18:2}$ constituted about 80% of the total fatty acids (table 4). The saturated fatty acids C_{16} and C_{18} were about 15%. Remaining fatty acids represented less than 5%. The fatty acid profile of the macrophage phospholipids was qualitatively comparable to those obtained by temperature programmed GLC analysis reported for mice resident peritoneal macrophages (Leslie *et al.*, 1985). The macrophage phospholipids in all the groups (figure 4) comprised of low levels of $C_{18:1}$ and $C_{18:2}$ (less than 12%, table 5), though they were abundantly present in the diet (table 4). This is quite intriguing as macrophage fatty acids are known to be modulated by dietary fats (Leslie *et al.*, 1985). The total saturated fatty acids (table 6) were 14–18% in the 3 wk control and 20% protein fed rats. It was higher (26%) in the 0 wk control and protein restricted groups. Macrophage phospholipids isolated from the 3 wk control and 20% protein fed rats contained very high amounts of long chain polyunsaturated fatty acids (PUFA) compared to the values

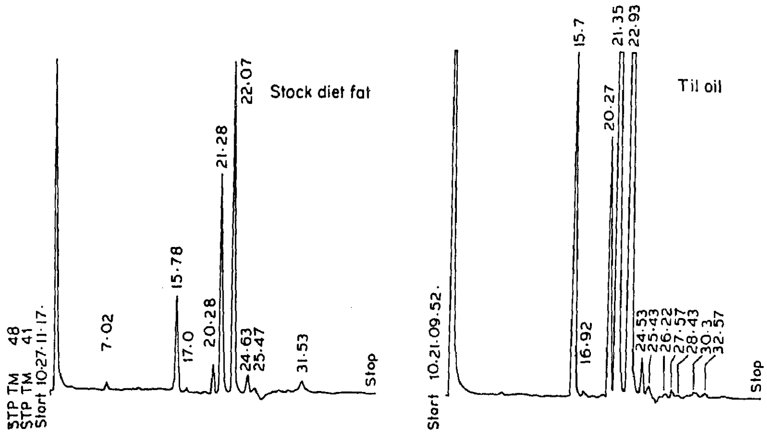

Figure 3. Separation of dietary fatty acids. Dietary fats were directly transesterified with methanolic HCl and total fatty acid methyl esters were analysed by GLC as described under methods. The major fatty acid peaks were identified as C_{16}(15), C_{18}(20), $C_{18:1}$(21), $C_{18:2}$(22), $C_{18:3}$(24) and $C_{20:1}$(26). The numbers in parentheses represent their retention time in min.

Table 4. Fatty acid composition of dietary lipids.

Fatty acids	Til oil	Stock diet fat
	% wt. of total fatty acids	
C_{12}	ND	⩽0·01
C_{16}	8·86	13·48
$C_{16:1}$	0·12	⩽0·01
$C_{18:0}$	5·79	3·85
$C_{18:1}$	38·42	30·66
$C_{18:2n\text{-}6}$	43·35	47·23
$C_{18:3n\text{-}3}$	1·52	2·35
$C_{20:1n\text{-}9}$	0·71	1·11
$C_{20:3n\text{-}9}$	0·14	ND
$C_{20:4n\text{-}6}$	0·36	ND
$C_{22:1n\text{-}9}$	0·19	⩽0·01
$C_{20:5n\text{-}3}$	0·05	1·287
C_{24}	0·30	ND
$C_{24:1}$	0·16	ND

Stock diet fat was obtained by Soxhlet extraction for 18 h using petroleum ether (B.Pt. 60–80°C). The fats were directly transesterified and FAME analysis done by GLC as described in the methods.
ND, Not detected.

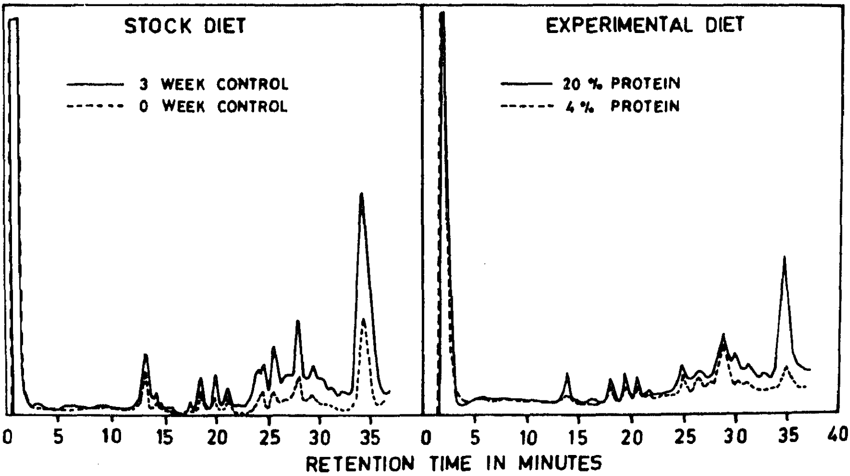

Figure 4. Fatty acid profile of macrophage phospholipids. Macrophage total phospholipids (100–150 μg/sample) eluted from TLC plate were transesterified and FAME analysis done by GLC. The profiles obtained with the 4 different groups were compared. The retention time for the major fatty acids identified in the samples in min (given in the parentheses) were as follows: C_{16}(14–15), C_{18}(19–20), $C_{18:1}$(21), $C_{18:2}$(22), $C_{18:3}$(24), $C_{20:1}$(26), $C_{20:3}$(27), $C_{20:4}$(28), $C_{20:5}$(30), C_{24}(31), $C_{22:5}$(32) and $C_{22:6}$(34–35).

reduced in the 0 wk control and protein restricted groups, compared to the other two groups (table 5). These values are more similar to that reported for the non elicited macrophage phospholipid fatty acids (Leslie *et al.*, 1985). The macrophages isolated from the protein deficient group contained significantly higher arachidonic

ble 5. Fatty acids of macrophage phospholipids.

	Control (wk)		Protein (%)	
ty acids	0	3	20	4
	% wt. of total fatty acids			
6	17·08 ± 3·84	10·31 ± 0·62	13·98 ± 1·76	16·61 ± 7·17
8	6·72 ± 1·4	3·78 ± 0·68	4·99 ± 1·88	6·73 ± 1·26
:1	5·42 ± 1·9	4·04 ± 1·09	5·18 ± 0·68	6·66 ± 2·26
:2n-6	7·07 ± 1·89	5·56 ± 1·69	4·22 ± 1·35	7·0 ± 0·22
:3n-3	3·92 ± 1·74	1·22 ± 0·49	1·21 ± 0·56	2·88 ± 0·58
:1n-9	2·07 ± 1·13	1·29 ± 0·56	1·66 ± 1·6	3·42 ± 1·06
:3n-9	4·35 ± 0·9	6·96 ± 2·75	4·05 ± 0·97	6·9 ± 2·3
:4n-6	12·27 ± 4·17	10·51 ± 2·15	14·31 ± 2·85	18·19 ± 2·0
:1n-9	2·78 ± 0·87	1·38 ± 0·56	1·19 ± 0·17	2·85 ± 0·97
:5n-3	14·30 ± 4·76	12·27 ± 1·42	11·62 ± 3·97	11·79 ± 3·29
4	3·00 ± 1·08	ND	ND	3·22 ± 0·39
:5n-3	5·65 ± 3·78	1·15 ± 0·68	ND	3·12 ± 1·57
:6n-3	15·34 ± 4·46	42·42 ± 3·52	37·53 ± 6·11	10·54 ± 1·39

ues are mean ± SD of 4 sets of samples per group, analysed in duplicates. Each experimental sample sisted of lipids extracted from 5 rats. Data are reported as per cent composition of the total ntifiable fatty acids.

d ($C_{20:4n\text{-}6}$; $P \leqslant 0{\cdot}013$) than the other 3 groups. The unsaturation index and saturation index/saturated ratio were much lower in the 0 wk and protein tricted groups (table 6). The n6/n3 ratio was higher in these two groups.

The long chain PUFA are ubiquitous and essential constituents of structural ids. They are derived from their precursors $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ by sequential saturation and chain elongation, mediated by Δ^6, Δ^5 and Δ^4 desaturates olman, 1986). Protein and essential fatty acid deficiency induced changes in their ivities in rat livers have been observed (De Tomas *et al.*, 1980). Recently Narce *al.* (1988), have shown that in growing rats, on a low protein diet, the fall in patic Δ^6 and Δ^5 desaturase activities lead to reduced formation of C_{20} and C_{22} ty acids. However the changes were reversed on balanced refeeding, implicating em to be direct effects of protein deficiency. Results presented here indicate the sence of active desaturases in macrophages, warranting separate studies on the synthesis of PUFA from their labelled precursors in protein restriction to nclusively assess the impact on these enzymes. DHA is synthesised by the ngation of C_{20} fatty acid precursors followed by the action of Δ^4 desaturase. Its luced levels in the protein deficient and 0 wk group suggest reduction in the ivity of this enzyme. Functionally active tissues such as brain and retina euringer and Connor, 1986) are rich in DHA. It is selectively incorporated into eolar macrophages (Huterer and Wherrett, 1985) and peritoneal macrophages slie *et al.*, 1985) in rats maintained on diets enriched in fish oil or n-3 fatty acids.

The importance of long chain PUFA as eicosanoid precursors is now well ognized. Macrophages abundantly synthesize prostaglandins and leukotrienes ich have potent bioregulatory roles (De Maroussem *et al.*, 1985). Prostaglandins E_1 and PGE_2 which are derived from n-6 fatty acid exhibit negative control in

Table 6. Classification of macrophage fatty acids.

	Control (wk)		Protein (%)	
Fatty acid class	0	3	20	4
Saturated (C_{16}, C_{18}, C_{24})	26·8	14·1	16·0	26·6
Unsaturated ($C_{18:1}$, $C_{18:2}$, $C_{18:3}$)	16·4	10·8	9·9	16·5
Long chain PUFA eicosaenoid series ($C_{20:1}$, $C_{20:3}$, $C_{20:4}$, $C_{20:5}$)	33·0	31·0	31·6	40·6
Docosaenoid series ($C_{22:1}$, $C_{22:5}$, $C_{22:6}$)	23·7	44·9	38·7	16·5
Unsaturation index[1]	290	406	372·7	266·9
Unsaturation index[2] [Saturated]	10·82	28·83	19·6	10·04
[Unsat]/[Sat][3]	2·73	6·1	4·27	2·75
Double bond indices[4] $\frac{n6}{n3}$	0·31	0·163	0·23	0·59

The data reported above is thc sum of the mean of fatty acids (% wt. of total fatty acids) represented in parentheses under each class.

The calculation of different indices was done as follows.

1. Unsaturation index (USI) = Σ(% of unsaturated fatty acid × no. of double bonds).
2. $$\frac{\text{Unsaturation index}}{[\text{Saturated}]}=\frac{\text{USI}}{\Sigma\,\%\text{ saturated FA}}.$$
3. $$[\text{Unsat}]\ [\text{Sat}]=\frac{\Sigma\,\%\text{ unsaturated FA}}{\Sigma\text{saturated FA}}.$$
4. $$\frac{n6}{n3}\text{ ratio}=\frac{\Sigma\,n6\text{ FA}\times\text{double bonds}}{\Sigma\,n3\text{ FA}\times\text{double bonds}}.$$

This regulation is essential in the prevention of pathophysiological states and may be affected in the protein deficient and 0 wk groups which contain higher n-6 fatty acids. EPA is known to be formed by the retroconversion of DHA in humans (Fischer *et al.*, 1987) and bovine endothelial cells (Hadjiagapion and Spector, 1987). Possibly in macrophages DHA may serve as an endogenous storage form of EPA.

By virtue of its disordering effect a role for DHA has also been envisaged in maintenance of membrane fluidity and associated functions (Conroy *et al.*, 1986). Observed changes in DHA levels could effect cell membrane physical properties. These parameters affect protein conformation and lipid protein interactions (Stubbs and Smith, 1984) which could reflect on the enzymes and receptor functions of macrophages. Long chain PUFA also have a role in macrophage activation and act as potent stimulators of their respiratory burst (Bromberg and Pick, 1983) and cytotoxic potential (Schlager *et al.*, 1983). Distortions in these functions have been observed in macrophages isolated from protein deficient rats (J. P. Machaiah, unpublished results).

The results presented here suggest that critical levels of macrophage lipid constituents are essential for optimum immune response. This is disturbed in protein deficient and young rats. Earlier observations have reported suboptimum mune response in neonates (Chandra, 1984). Protein restriction has apparently own regulated the thioglycollate elicitation of macrophages to an extent mparable to that observed in young rats. Observed variations in macrophage id components in these groups could profoundly influence its membrane fluidity, ceptor activity and eicosanoid metabolism; these parameters regulate its key munologic functions pertaining to phagocytosis and cytotoxicity.

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ect of epinephrine on the *in vitro* biosynthesis of fibrinogen

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Abstract. In an *in vitro* rat liver slice incubation system, the synthesis of fibrinogen, when measured by immunoprecipitation technique was stimulated in the presence of epinephrine. An increase in poly $(A)^+$ RNA content of the liver slice was also observed after epinephrine treatment. This *in vitro* experiment demonstrated that epinephrine stimulated *de novo* synthesis of fibrinogen by acting directly on the liver.

Keywords. Epinephrine; rat liver slice; fibrinogen; poly $(A)^+$ RNA; stimulation; *de novo* synthesis.

oduction

plasma fibrinogen level is elevated during certain pathophysiological conditions atterjee *et al.*, 1978; Kampschmidt and Upcharch, 1974). At the molecular level defined physiological substance or mediator has been identified to explain the ation of fibrinogen level. However, a nonphysiological substance, turpin oil after inistration to rats showed higher plasma level of fibrinogen (Bouma *et al.*, 5). The administration of biogenic amines to rats also elevated the level of ma fibrinogen (Sur *et al.*, 1979). Inhibitors of monoamine oxidase and abolites of amines failed to show such stimulatory response (Sarkar *et al.*, 1984). *e novo* synthesis of fibrinogen was suggested for this elevation of plasma nogen level since higher incorporation of [^{14}C]-amino acid into plasma nogen was abolished by actinomycin D (McKenzy *et al.*, 1966; McKenzy and ler, 1968; Roy *et al.*, 1985a). The liver, being the site of fibrinogen synthesis nn *et al.*, 1951; Miller *et al.*, 1951, 1963), was investigated for the incorporation [^{14}C]-orotic acid into polysomal RNA, the level of DNA-dependent RNA merase activity and also for the content of fibrinogen-specific functional mRNA the animals treated with amines and untreated one. Each of these parameters wed distinct elevation for amine treated animals only (Roy *et al.*, 1985a, b). All e *in vivo* studies led to the suggestion that the amine itself is responsible for the ulatory synthesis of fibrinogen.

o answer the question whether the biogenic amine acts directly on the liver, an ated rat liver slice incubation system was designed as an *in vitro* fibrinogen hesising system. The present study demonstrated that the effect of the amine direct on the liver and specific for stimulated synthesis of fibrinogen.

terials and methods

pinephrine, α-amino caproic acid, aptroteinin, amino acids were purchased from

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whom all correspondence should be addressed.

Sigma Chemical Co., St. Louis, Missouri, USA, Oligo (dT)-cellulose was from Collaborative Research, Lexington, Massacusetts, USA and casamino acid was procured from Difco Laboratories, USA. [^{14}C]-Chlorella hydrolysate and [^{32}P] (carrier free inorganic phosphate) were obtained from Bhabha Atomic Research Centre, Bombay. All other reagents used were of analytical grade.

Experimental procedure

Incubation of liver slices: Livers from rats (Wister albino, IICB inbred) pretreated with reserpine (Vijayalakshmi *et al*., 1978) 24 h prior to sacrifice were perfused for making slices of 0·3 mm thick (Ballard and Oliver, 1963). About 0·2 g of this was primed with epinephrine by 30 min incubation at 37°C in 2 ml of Kreb's Ringer bicarbonate buffer solution having 20 μg/ml epinephrine. Then the slices were washed with the same buffer twice and the washings were discarded. This was then grouped into experimental and control. Two ml of Kreb's Ringer bicarbonate buffer solution having trasylol (500 KIU/ml), α-amino caproic acid (1 mM), [^{14}C]-chlorella hydrolysate (20 μCi) and amino acid mixture at half the concentration used by Morgan (1969) was used for both the sets. Experimental samples received 20 μg per ml of epinephrine, while controls received only vehicle. The slices were incubated at 37°C in an atmosphere of air and CO_2 (95 and 5%). The medium from each flask was collected after specified period of incubation, clarified and used for estimation of radiolabelled fibrinogen content by immunoprecipitation technique. For measuring poly $(A)^+$ RNA synthesis, the liver slices were labelled with ^{32}Pi (100 μCi/5 ml medium).

Immunoprecipitation of fibrinogen: The monospecific antifibrinogen antibody was prepared by immunoaffinity chromatography (Bouma and Fuller, 1975). Elution was effective with 3 M ammonium thiocyanate, which was readily eliminated from the eluate by quick dialysis and the antibody was stored at −20°C until use. The incubation medium collected and clarified as stated above was treated for 10 min with formalinised heat killed *Staphylococcus aureous* (Cown-1 strain) bacterial suspension (10%) for its pre-cleaning (Martial *et al*., 1977). The soup, after separation of bacteria was incubated with 3-fold excess of antibody (with respect to the approximate fibrinogen content of the soup) for 3 h at room temperature and then at 4°C overnight. The immune complex so developed was then estimated after Kessler (1975). Unlabelled pure rat fibrinogen was added in excess (100 μg) to the clarified and precleaned incubation medium prior subjecting it to immunoprecipitation and then the radioactivity in the immune complex was estimated.

Protein was estimated using bovine serum albumin as standard (Lowry *et al*., 1951).

Poly $(A)^+$ RNA preparation and determination: Approximately 1 g of the incubated slices from [^{32}P] containing incubation system was used in each batch for RNA preparation. Polysomal RNA was prepared according to Palmiter (1974) and then subjected to fractionation over oligo (dT)-cellulose affinity column into bound and unbound fractions (Aviv and Leader 1972; Nickerson and Fuller 1981). The content of RNA was measured following the procedure of Ceriotti (1955) using yeast RNA as standard.

sults and discussion

this *in vitro* incubation system, the incorporation of [^{14}C]-Chlorella protein lrolysate into TCA precipitable material increased linearly up to 4 h of ubation. But [^{32}P] incorporation into TCA insoluble form showed a linear rease only up to 2 h of incubation. Hence incubation medium for estimation of inogen content was collected after 2 h of incubation and liver slices for RNA paration was taken after 1 h of incubation.

'he synthesis of fibrinogen both in the presence and absence of epinephrine in liver slice incubation systems was determined by immunoprecipitation method ng incubation medium. The labelled fibrinogen obtained after the liver slice ubation with epinephrine was nearly 62% higher than that obtained from trols (table 1). Table 1 further indicated that total protein synthesis, was vever, slightly reduced in the presence of epinephrine. The *in vitro* synthesised inogen was displaced in a quantitative manner when unlabelled pure rat inogen was added to the incubation medium prior to the addition of anti-inogen antibody for immunoprecipitation. In the presence of excess unlabelled inogen the radioactivity in the immune-complex was nearly identical to the kground value (data not shown). Therefore, this *in vitro* experiments demon-ited that the amine acted directly on the liver to give the stimulated synthesis of inogen. The effect of amine is apparently specific at least for fibrinogen. Epine-ine, at a dose of 20 μg/ml stimulated maximally the incorporation of radio-ivity into fibrinogen (figure 1).

Table 1. *In vitro* effect of L-epinephrine on fibrinogen synthesis.

	cpm/mg of protein (extracellular)		
	Total protein	Immuno-precipitation	Stimulation (%)
Control	18,049 ± 62	296 ± 35	—
L-Epinephrine (20 μg/ml)	14,823 ± 34	480 ± 28*	62

Values are mean ± SE of 6 determinations.

*$P < 0.01$ *vs* control.

The immunoprecipitate was washed with buffer A (150 mM NaCl, 5 mM EDTA, 50 mM Tris · HCl pH 7·4 and 0·02% NaN_3). This was then heated with 200 μl of 3% SDS, 10% glycerol at 90°C for 5 min. Then the centrifugation separated the supernatant containing labelled antigen, 50 μl aliquot of this was used for radioactivity determination.

ly(A)$^+$ RNA synthesis

e polysomal RNA synthesis of the liver slices for both the control and epine-rine containing incubation systems was estimated and the results are shown in le 2.

'he Poly (A)$^+$ RNA isolated from epinephrine-treated liver slices showed 37% her incorporation of [^{32}P] than the controls. The oligo (dT)-cellulose unbound ction of polysomal RNA as well as total polysomal RNA showed only a marginal rease in amine treatment compared to controls. It is possible that the increased

Table 2. Effect of L-epinephrine on [^{32}P] incorporation in polysomal RNA, oligo (dT)-cellulose bound and unbound fractions of polysomal RNA (*in vitro*).

	Polysomal RNA cpm/mg of RNA Mean ± SE	Unbound		Bound	
		cpm/mg of RNA	Stimulation (%)	cpm/mg of RNA	Stimulation (%)
Control	4192 ± 24	3806 ± 41	—	1054 ± 25	—
L-Epinephrine	4402 ± 56	4075 ± 92	—	1450 ± 63	37·5

Each value represents the mean ± SE of 6 determinations; liver slice incubation was described in materials and methods. The experimental details are given in text.

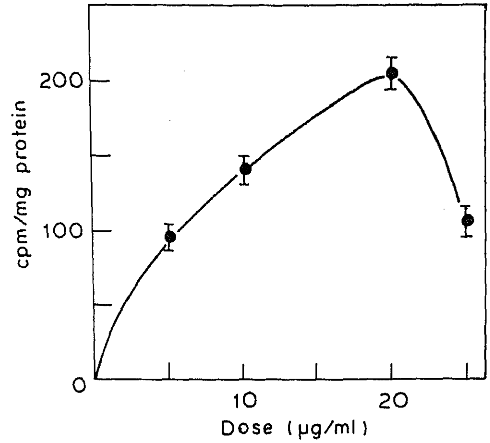

Figure 1. Dose response curves of L-epinephrine on fibrinogen synthesis in rat liver slice system.

poly (A)$^+$ mRNA synthesis in epinephrine treatment may include fibrinogen mRNA as well.

However, it may be pointed out that the extent of stimulation for both the fibrinogen and poly(A)$^+$ RNA was considerably low in this *in vitro* system compared to *in vivo* effects. Such lower value might arise from the loss of organisational integrity of liver, and certain cellular damage during its slicing. In conclusion, this *in vitro* study demonstrated that direct action of epinephrine on the liver is responsible for stimulated synthesis of fibrinogen.

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istochemical observations on the crown skin of male baya: Lipids, pase and phosphomonoesterases

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Abstract. Histochemical studies on localisation of lipids, lipase and phosphomonoesterases in the crown skin of male baya during non-breeding and breeding seasons were carried out. The results indicated a high turnover rate for lipid synthesis and its utilisation, increased acid and alkaline phosphatase activities in the crown skin of breeding male baya as compared to that in non-breeding season. It is surmised that crown skin of male baya becomes metabolically hyperactive during breeding season aiding processes such as cell growth and proliferation, keratinization and production of coloured feathers related to breeding.

Keywords. Lipids; lipase; acid phosphatase; alkaline phosphatase; crown skin; baya weaver bird.

troduction

1e male baya weaver bird, *Ploceus philippinus*, in breeding season (April–October) oults into a bright golden yellow plumage on its crown and upper breast regions om the dull brown henny non-breeding type plumage. It is believed that the cyclic ianges in the plumage patterns in male baya are under the control of cyclic tivity of pituitary gland (Thapliyal and Saxena, 1961). However, the nuptial umage forming crown skin of male baya has been shown to be a potential target gion for sex-steroid metabolism (Kotak and Menon, 1982). Further, the crown in during breeding season showed structural variations in terms of overall crease in thickness, mucopolysaccharide and protein content from that observed non-breeding season (Narasimhacharya, 1987) which coincided with increasing vels of both plasma luteinizing hormone and testosterone (Narasimhacharya *et al.*, 88). Keeping these findings in view, the physiological and metabolic role of lipids, pase and phosphomonoesterases in nuptial plumage formation in male baya were amined histochemically in crown skin during non-breeding and breeding seasons.

aterials and methods

dult male weaver birds were shot in Vallabh Vidyanagar from free-living pulations during breeding (April–October) and non-breeding (November–March) asons. The crown skin of the birds was immediately trimmed free of feathers, cised and fixed on the chucks of an AO–cryocut microtome at −20°C. 10–12 μm ick sections were processed for histochemical localization of total and acidic lipids

(Pearse, 1968), lipase (Gomori, 1952), acid and alkaline phosphatases (Bancroft and Stevens, 1977).

Results

Total lipids

The amount of total lipids increased considerably in the crown skin of breeding male baya (figure 1B) as compared to that of non-breeding male (figure 1A). While the epidermis and feather follicles did not register an increase in lipid accumulation, dermis particularly showed a greater accumulation of lipids in breeding season.

Acidic lipids

A moderate increase in acidic lipid content was discernible in crown skin during breeding season (figure 1D) from non-breeding season. The feather follicles and dermal matrix showed a higher acidic lipid content as compared to those in non-breeding season (figure 1C).

Lipase

The crown skin of non-breeding males registered a weaker enzyme response in epidermis, dermis and feather follicles (figure 1E) in contrast to the intense enzyme activity in these regions of the integument of breeding males (figure 1F).

Acid phosphatase

The crown skin in non-breeding season revealed a moderate acid phosphatase activity in epidermis and a very little activity in dermis and feather follicles (figure 2A). During breeding season, the crown skin exhibited an intense activity of the enzyme in epidermis, feather follicles and dermal compartments (figure 2B).

Alkaline phosphatase

The pattern of alkaline phosphatase activity during breeding and non-breeding seasons was more or less similar to that seen in case of acid phosphatase. A greater intensity of alkaline phosphatase was noted in epidermis, dermal regions including follicles during breeding season (figure 2D) as against a weak activity of this enzyme in non-breeding season (figure 2C).

Discussion

The normal metabolic processes, development of feathers and epidermal keratinization are known to be largely dependent on the lipid synthesizing ability of the integument itself (Flaxman, 1972; Matoltsy, 1969). A greater distribution of total

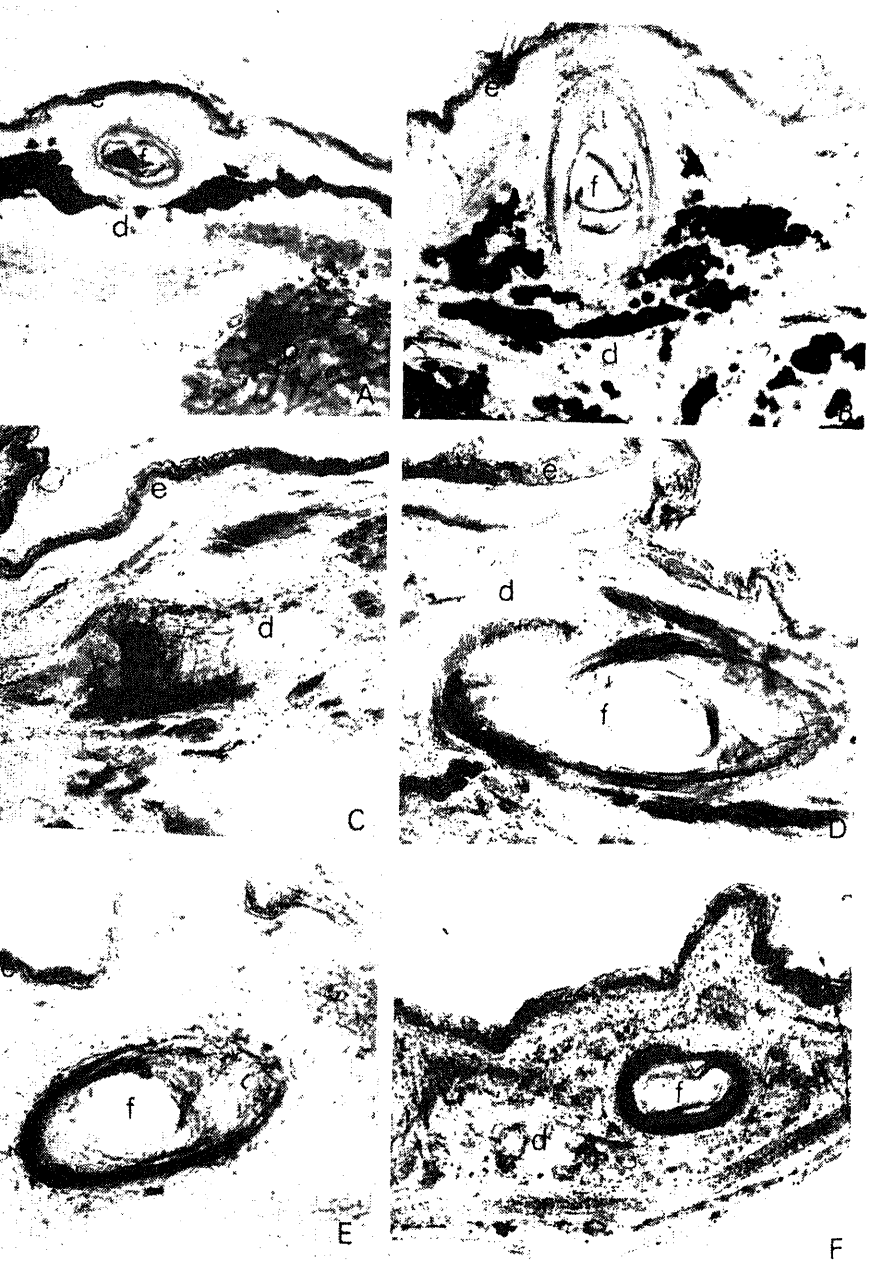

Figure 1. Vertical sections of crown skin of male baya showing histochemical distribution of total and acidic lipids and lipase during non-breeding and breeding seasons. **A.** Total lipid localization in the crown skin in non-breeding season (× 120). **B.** The crown skin of male baya in breeding season depicting an increased total lipid content (× 120). **C.** Distribution pattern of acidic lipids in the non-breeding baya crown skin (× 120). **D.** Localization of acidic lipids in breeding baya crown skin (× 120).

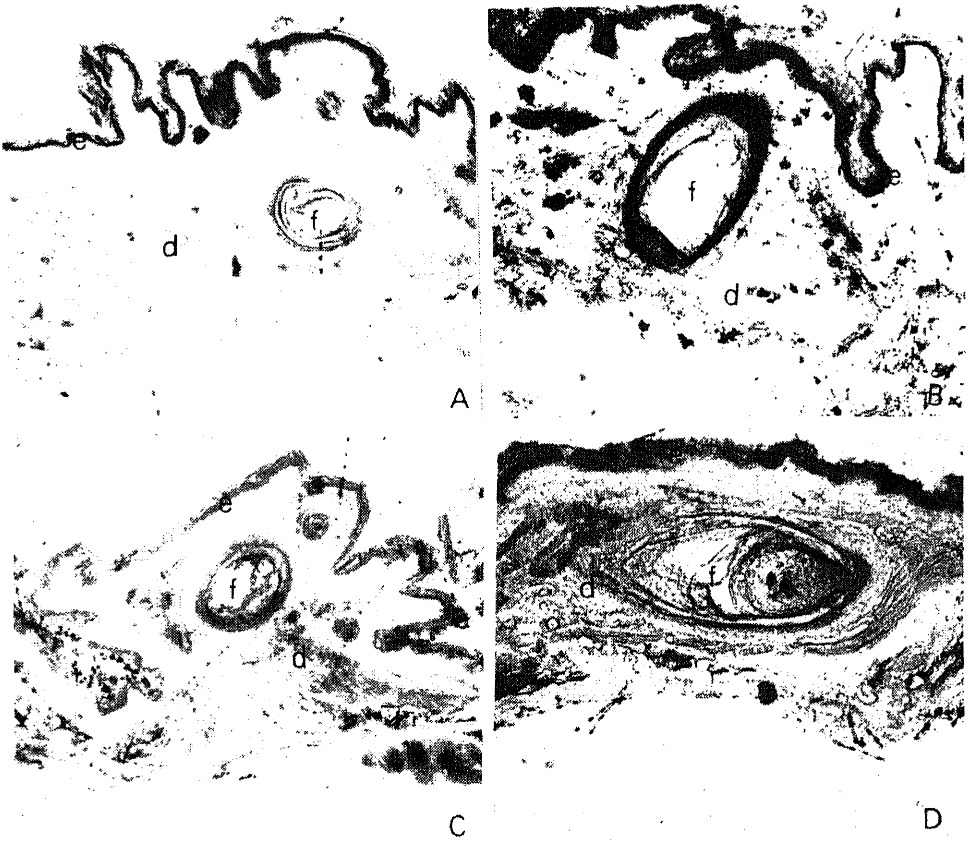

Figure 2. Vertical sections of male baya crown skin during non-breeding (**A, C**) and breeding (**B, D**) seasons. **A.** Localization pattern of acid phosphatase in non-breeding season (× 120). **B.** Activity of acid phosphatase during breeding season (× 120). **C.** Histochemical localization of alkaline phosphatase in a non-breeding baya crown skin (× 120). **D.** Alkaline phosphatase activity and its distribution in the crown skin of male baya in breeding season (× 120).
(d, Dermis; e, epidermis; f, feather follicle; p, pith tissue).

and acidic lipids in crown skin of breeding baya (figure 1B,D) is suggestive of crown skin's increased ability to synthesize the lipids as compared to that of non-breeding males (figure 1A, C). Such an increase in lipid content of the integument was also reported in pigeon, during different phases of feather development (Shah and Menon, 1972). Since the development of feather and its emergence is a phase of active cell proliferation and differentiation requiring higher amounts of energy, active lipid metabolism is considered essential as lipids are chief source of energy for laying down the structural frame work of feathers (Bell and Thathachari, 1963). Besides being rich energy source, acidic lipids are also incorporated as structural components of feather forming epithelial cells (Shah and Menon, 1972). Further the proposed role of lipids in keratinization of epidermal and follicular cells (Bell and Thathachari, 1963) also suggests that an increased lipid content in the crown skin in breeding season could cause an increased keratin-synthesis (Narasimhacharya, Evidence for lipid utilisation comes from the fact that the crown skin of ing males registered an increased lipase activity (figure 1F) as compared to reeding males (figure 1E). The functional similarities between lipase and ses (Shah and Menon, 1972) in hydrolysing lipid moities aiding the process of nization during feather development and differentiation, seems to be pertinent present case too, as during breeding season male baya develops an entirely et of feathers related to breeding. Increased lipid synthesis and its subsequent tion may also imply a carotenoid-linked lipid turnover towards nuptial age formation, for, it is well known that lipids help dissolve and store the y and/or metabolically derived pigments like carotenoids (Spearman and y, 1985). As the lipids get increasingly metabolised, the dissolved pigments get ited in growing feathers (Ralph, 1969).

osphomonoesterases (acid and alkaline phosphatases) have been demonstrated avian skin and correlated with organogenesis of cutaneous appendages and al metabolic processes of integument (Shah and Menon, 1971, 1974). The nce of acid phosphatase in different layers of integument has been considered important for lipid metabolism, synthesis of specific proteins and also hydrate metabolism (Mishima, 1964; Shah and Menon, 1971). Evidently, an se acid phosphatase activity in crown skin (figure 2B) during breeding season njunction with increased lipid content (figure 1B, D), epidermal and dermal plasia, increased protein and mucopolysaccharide content and, a high rate of inization (Narasimhacharya, 1987) indicate a possible role played by acid hatase in mediating these activities. Even though the activity of alkaline hatase in the crown skin of breeding baya was not as intense as that of acid hatase (compare figure 2B and D), its increased activity from non-breeding n (figure 2C) indicates its role in cell growth and differentiation, epidermal cell eration, biogenesis and metabolism of lipids and transport of metabolites ers, 1960; Shah and Menon, 1974). Increased presence of this enzyme in rmis and feather follicles is further suggestive of its role in laying down the tural elements of tissues, increased feather growth and integumentary polysaccharides (Kroon, 1952). Thus an increased lipid accumulation mpanied by an increased lipolytic activity with a simultaneous increase in the ties of both acid and alkaline phosphatases in the crown skin of male baya in ling season, probably indicate that this skin region is metabolically well-ped system that not only assists in proliferation of epidermal and dermal ponents but also in the emergence of nuptial feathers related to breeding.

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Effects of postnatal treatment with naloxone on plasma gonadotropin, prolactin, testosterone and testicular functions in male rats

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Abstract. Opioid peptides are implicated in the control of gonadotropin and prolactin secretion. The role of opioid antagonist naloxone and its effects on plasma gonadotropin, prolactin, testosterone levels and testicular hyaluronidase, acid phosphatase, [^{3}H]uridine and thymidine incorporation, RNA, DNA and protein concentrations were evaluated in rats after administration of naloxone beginning day 1 through 21 and autopsied on 45, 60 and 90 days of age. Plasma gonadotropin and testosterone levels were significantly elevated after naloxone treatment. Testicular hyaluronidase and acid phosphatase activity increased till 60 days post treatment and declined thereafter. Concentrations of RNA and protein did not change significantly but the concentration of DNA declined at 45 and 60 days of age. These results suggest that endogenous opioid peptides exert regulatory influence on gonadotropin secretion which in turn control the testicular function in the male rat.

Keywords. Opioid peptides; pituitary; plasma hormone levels; naloxone; neonatal rats.

Introduction

Narcotics and opioid peptides are known to decrease the activity of hypothalamic pituitary gonadal axis where as narcotic antagonists increase serum LH in rodents and humans (Cicero *et al.*, 1976, 1977; Miller *et al.*, 1986). It has also been shown that opioid peptides modulate luteinizing hormone (LH) secretion during sexual maturation in the rat (Blank *et al.*, 1979). Naloxone, an opioid receptor antagonist competitively inhibits the effects of opioids on LH secretion. However, treatment with morphine, naloxone and morphine plus naloxone do not modify the secretion of follicle stimulating hormone (FSH) in normal and castrated male rats (Piva *et al.*, 1986). The results with opioids and of their antagonists on FSH secretion is still not clear. Naloxone could increase LH levels or block testosterone's negative feedback control of LH by displacing the androgen from its receptors in the brain or pituitary (Cicero *et al.*, 1979). Lee and Loh (1975) have recently noted from [^{3}H] morphine incorporation studies that DNA is one of the narcotic receptors and chronic morphine treatment increases the chromatin template activity. In contrast, decrease of chromatin template activity is also noted (Hodgson *et al.*, 1971). Chronic morphine treatment in mice however, inhibited incorporation of labelled uridine and thymidine and hence RNA and DNA synthesis (Datta and Antopol, 1971). The present experiments were designed to evaluate the effects of opioid antagonist naloxone treatment in neonatal rats beginning day 1 of birth. Concentration of DNA, RNA and protein, acid phosphatase and hyaluronidase enzyme activity in the testes and plasma levels of gonadotropin, prolactin and testosterone were evaluated on 45, 60 and 90 days post-treatment.

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Abbreviations used: LH, Luteinizing hormone; FSH, follicle stimulating hormone; RIA, radioimmunoassay; EOP, endogenous opioid peptides; LHRH, luteinizing hormone releasing hormone; TSH, thyroid stimulating hormone.

Materials and methods

Female Wistar rats with pups were maintained in the School animal house facility under a 14 h light and 10 h dark regimen. They were fed on standard rat pellets purchased locally and with drinking water *ad libitum*. Day of birth was assigned as day 1 and pups were kept with their mother till day 21 postpartum. On day 22 they were weaned, males and females were kept in separate cages till they attained the age of 45, 60 and 90 days respectively.

Naloxone (2 mg/kg) was injected subcutaneously on alternate days from day 1 till 21 of age. Controls received equal volume of saline. Body weights of the animals were recorded during the treatment period as well as on day 45, 60 and 90 respectively, before they were decapitated. Testes, seminal vesicles and ventral prostate were dissected out, kept at 4°C, cleaned and weighed to the nearest 0.2 mg on a torsion balance. Trunk blood was collected and plasma was separated by centrifugation at 4°C and kept frozen for later assay of hormones. Pituitary and plasma LH, FSH, prolactin and testosterone were measured by radioimmunoassay (RIA). RIAs were carried out, using kits obtained from NIH-NIAMDD, by a double antibody technique and the data were expressed as ng/ml of plasma with reference to RP-1 reference standard for rat-LH, FSH and prolactin. Testosterone levels were measured by steroid hormone RIA (courtesy Diagnostic System Inc, Houston, USA) and the results are expressed as ng/ml of plasma.

Testes were decapsulated and a part of the testicular tissue was incubated 2 μCi [^{3}H] thymidine (sp. activity 17,000 mCi/mmol) in Dulbecco's Eagle medium containing 40 mM HEPES (pH 7·4), 1% bovine serum albumin (BSA) and 1% glucose. Incubation was carried out at 34°C for 90 min. The reaction was stopped by placing the tissues in ice, washed with cold distilled water and processed for nucleic acid extraction by the procedure standardised in the laboratory (Mistry and Vijayan, 1985; Prasad, 1986).

An aliquot of the sample was taken and put in Bray's scintillation fluid and counted in a Beckman LS 1800-counter with 40% efficiency for tritium. The above procedure was repeated for studying the incorporation of [^{3}H] uridine (sp, activity 6,000 mCi/mmol) into RNA in the testis. Two μCi of uridine was added to the medium containing the tissue and incubation was carried out for 2 h (Mistry and Vijayan, 1985).

Assay of hyaluronidase activity

Testicular hyaluronidase activity was assayed according to the procedure of Males and Turkington (1971) and Doak and Zahler (1979) with slight modifications. Ten μl of the enzyme preparation was used and free N-acetylglucosamine (NAGA) end groups produced by hyaluronidase action were estimated as described earlier (Prasad and Vijayan, 1986, 1987).

ay of acid phosphatase activity

d phosphatase activity was assayed according to the method of Henrickson and ver (1972) and Pertulla *et al.* (1986) as standardised in the laboratory. Testicular ue was homogenised in 0·3% Triton-X100. Acetate buffer (pH 6) containing yme samples were incubated with 0·1 mM cobalt chloride. After incubating for nin, 2 μmol of phosphatase substrate was added. Substrate was freshly prepared the reaction terminated with 0·1 N NaOH after 1 h and the liberated *p*-nitro- nol (PNP) was estimated at 420 nm against the enzyme blank.

stimations of DNA, RNA and protein were carried out according to standard cedures routinely adopted in the laboratory (Mistry and Vijayan, 1985; Prasad, 6).

tistics: Group means were compared by analysis of variance followed by ncan's new multiple range test with $P<0\cdot5$ required for significance.

sults

uitary LH and FSH concentrations did not differ significantly in the control rats 45 and 60 days of age. However, naloxone treatment produced significant $<0\cdot05$) increase in both LH and FSH at 45 and 60 days of age while there was no preciable difference in gonadotropin concentration at 90 days of age. Pituitary lactin concentration did not show any significant change at any age studied ble 1).

Table 1. Gonadotropin and prolactin content in the pituitary gland of neonatal rats treated with naloxone.

	Age at autopsy (days)		
Treatment	45	60	90
LH (μg/pit)			
Control	146·41 ± 14·62	148·45 ± 24·81	153·61 ± 32·90
Naloxone	254·82 ± 24·71**	214·20 ± 3·80*	140·62 ± 21·32
FSH (μg/pit)			
Control	141·32 ± 15·62	138·61 ± 17·01	94·60 ± 19·41
Naloxone	184·63 ± 16·13*	196·25 ± 21·40**	106·44 ± 14·22
Prolactin (μg/pit)			
Control	4·40 ± 0·20	4·70 ± 0·30	5·40 ± 0·30
Naloxone	4·70 ± 0·80	5·40 ± 0·10	6·20 ± 0·20

Naloxone 2 mg/kg was injected on day 1 through 21 once daily on alternate days. Controls received equal volume of saline.

Values are mean ± SEM for 6–8 rats per group.

Values $^*P<0\cdot05$, $^{**}P<0\cdot01$ *vs* control.

levels of LH at 60 ($P<0\cdot01$) and 90 days ($P<0\cdot001$) of age whereas increase in the levels of FSH in the plasma was constant at 60 and 90 days of age ($P<0\cdot01$) when compared to their respective control values (table 2).

Table 2. Plasma levels of gonadotropin, prolactin and testosterone at different ages after neonatal treatment with naloxone.

	Age at autopsy (days)		
Treatment	45	60	90
LH (ng/ml)			
Control	2·30 ± 0·20	3·00 ± 0·30	2·50 ± 0·30
Naloxone	3·70 ± 0·50*	7·00 ± 1·00**	9·18 ± 1·08***
FSH (ng/ml)			
Control	586·20 ± 38·20	672·60 ± 24·20	642·60 ± 78·40
Naloxone	984·70 ± 41·20**	1108·00 ± 102·00**	1204·40 ± 64·60**
Prolactin (ng/ml)			
Control	21·00 ± 2·10	16·80 ± 1·40	15·20 ± 3·00
Naloxone	28·20 ± 2·40	17·00 ± 1·80	18·10 ± 2·40
Testosterone (ng/ml)			
Control	1·0 ± 0·4	2·6 ± 0·6	3·2 ± 0·2
Naloxone	2·2 ± 0·2*	4·4 ± 0·4*	4·8 ± 0·4*

Naloxone 2 mg/kg was injected subcutaneously from day 1–21 on alternate days for one month in neonatal rats. Controls received equal volume of saline.

Values represent mean ± SEM for 6–8 animals.

Values $^*P<0\cdot05$, $^{**}P<0\cdot01$, $^{***}P<0\cdot001$ are significantly different from their respective control.

Plasma testosterone levels showed a gradual increase with age in control rats. Naloxone treatment however, induced significant increase ($P<0\cdot05$ *vs* control) in testosterone levels at all ages studied which correlated with the increase in plasma LH levels (table 2).

There was no significant difference in body weights between control and naloxone treated rats in any particular age group. Body weights however, increased with age in both control and naloxone treated rats. There was a marginal, but significant ($P<0\cdot05$ *vs* control) increase in the weight of the testes and seminal vesicle in rats treated with naloxone and autopsied on day 45. The weight of testes increased significantly ($P<0\cdot01$ *vs* control) at 60 days of age whereas the weight of the seminal vesicle and ventral prostate did not exhibit any significant difference when compared to those of the controls. At 90 days after naloxone treatment the weights of testes, seminal vesicle and ventral prostate did not differ significantly from control values though plasma testosterone levels were significantly higher in these rats (table 3).

The rate of [^{3}H] thymidine incorporation into DNA increased significantly in the testes of rats treated neonatally with naloxone and autopsied at 45 and 60 days of age ($P<0\cdot01$ *vs* control), whereas at 90 days of age there was no significant difference. Testicular hyaluronidase activity increased at 45 and 60 days of age while the acid phosphatase activity increased only in 45 days old rats whereas the activity of both the enzymes decreased significantly ($P<0\cdot01$ *vs* control) at 90 days of age (table 4). The DNA concentration however decreased ($P<0\cdot01$) at 45 and 60

Table 3. Weight of the body, testes and accessory reproductive glands at different ages after neonatal treatment with naloxone.

	Age at autopsy (days)		
Treatment	45	60	90
Body weight (g)			
Control	160 ± 10	180 ± 10	200 ± 10
Naloxone	150 ± 15	195 ± 15	220 ± 10
Testis (mg)			
Control	442 ± 46	812 ± 90	913 ± 104
Naloxone	640 ± 94*	1201 ± 77**	782 ± 105
Seminal vesicle (mg)			
Control	25·00 ± 4·30	83·00 ± 19·00	253·00 ± 41·00
Naloxone	51·00 ± 12·50*	67·00 ± 10·00	268·00 ± 37·00
Ventral prostate (mg)			
Control	35·70 ± 8·80	105·70 ± 8·20	293·00 ± 58·00
Naloxone	49·50 ± 13·80	107·70 ± 5·70	245·00 ± 141·00

Naloxone 2 mg/kg was injected on day 1 through 21 once daily on alternate days. Controls received equal volume of saline. Body weight is represented in g and weight of accessory reproductive glands and testis is represented in mg.

Values are mean ± SEM for 6–8 rats per group.

Values $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ *vs* control.

s of age. Concentrations of RNA and protein and $[^3H]$ uridine incorporation RNA in the testes of naloxone treated rats, however, did not exhibit any ificant alteration (table 4).

cussion

logenous opioid peptides (EOP) and their antagonists appear to influence the ase of hormones from the pituitary mediated *via* hypothalamus or *via* othalamic neurotransmitters. Norepinephrine and epinephrine have been gested as possible neurotransmitters mediating the action of opioid peptides ites *et al.*, 1977, 1979). Chronic administration of naloxone to neonatal rats eases pituitary as well as plasma LH and FSH levels, thus indicating an eased synthesis and release of gonadotropin under blockade of endogenous oid inhibition. As opioid peptides and their antagonists do not have a direct on on the pituitary (Weisner *et al.*, 1984) the increased synthesis and release may due to an alteration in the activity of luteinizing hormone releasing hormone RH) caused by naloxone directly or *via* neurotransmitters. It may also be due the continuous blocking effects of naloxone at the opioid receptors when ninistered chronically to neonatal rats. Days 2–7 postnatally is a highly critical e in the development of opiate receptors in the rat brain (Simon and Hiller, 8) and naloxone treatment during the early neonatal days could possibly modify interaction between endogenous opioids and their binding sites. Naloxone tment antagonizes the inhibitory tone of the opioid peptides on gonadotropin thus there are increased levels gonadotropin in the plasma till 90 days. ester *et al.* (1980) have shown simultaneous stimulation of LH and FSH

Table 4. Effect of neonatal treatment with naloxone on (i) *in vitro* [^{3}H] uridine and thymidine incorporation, (ii) activity of hyaluronidase and acid phosphatase and (iii) concentration of RNA, DNA and protein in the testes of rats at 45, 60 and 90 days of age.

	Age at autopsy (days)		
Treatment	45	60	90
Specific activity (DPM × 10^{-3}/mg RNA) of RNA			
Control	46·61 ± 4·60	42·15 ± 6·92	40·80 ± 4·05
Naloxone	40·65 ± 4·46	45·21 ± 2·39	46·08 ± 18·11
Specific activity (DPM × 10^{-4}/mg DNA) of DNA			
Control	46·34 ± 1·44	35·26 ± 3·24	29·60 ± 4·81
Naloxone	52·41 ± 2·16**	45·80 ± 2·82**	28·95 ± 0·99
Hyaluronidase[a]			
Control	0·61 ± 0·06	1·85 ± 0·06	1·69 ± 0·15
Naloxone	1·38 ± 0·19***	2·46 ± 0·16***	1·21 ± 0·15**
Acid phosphatase[b]			
Control	5·18 ± 0·48	5·46 ± 0·53	7·50 ± 0·83
Naloxone	7·36 ± 0·48**	6·13 ± 0·39	4·50 ± 0·43**
DNA μg/100 mg tissue			
Control	236 ± 23	355 ± 11	189 ± 19
Naloxone	138 ± 24**	249 ± 29**	168 ± 9
RNA μg/100 mg tissue			
Control	642 ± 19	563 ± 60	406 ± 63
Naloxone	661 ± 38	522 ± 70	391 ± 18
Protein mg/100 mg tissue			
Control	4·50 ± 0·14	5·20 ± 0·15	4·20 ± 0·25
Naloxone	4·20 ± 0·21	4·80 ± 0·31	4·20 ± 0·40

Naloxone 2 mg/kg was injected on day 1 through 21 once daily on alternate days. Controls received equal volume of saline.

Values are mean ± SEM for 6–8 rats per group.

Values *$P<0·05$, **$P<0·01$, ***$P<0·001$ *vs* control.

Activity of enzymes expressed as [a]μmol NAGA formed/mg/protein/h and [b]μmol PNP released/mg/protein/h.

secretion by naloxone. Presumably this effect is due to naloxone's antagonism of the actions of EOP at a common receptor in the hypothalamus or possibly at extra-hypothalamic sites in the brain. Thus EOP containing systems may play a physiologically relevant role in tonically inhibiting the release of LHRH and thus the disinhibition induced by naloxone results in a sudden discharge of LHRH.

Though there was not much difference in the concentration of RNA and protein per 100 mg tissue at 45, 60 and 90 days of age between experimental and control animals, the significant increase in the content of RNA and protein per testis and decrease in concentration of DNA at 45 and 60 days of age may possibly be due to significant increase in the weight of testes. The weights of ventral prostrate and seminal vesicles were less affected in 60 and 90-day old animals than in the 45-day old animals. Though the increase in gonadotropin levels were higher at 90 days of age than those in 60 days of age the magnitude of stimulatory effect on plasma testosterone levels was nearly equal. The failure of enhanced levels of gonadotropin timulate corresponding increase in testosterone levels may possibly be due to ensitization of LH receptors in the testes. This is consistent with the known nomena of negative regulation of receptors in the target organ of the rat testes tt *et al.*, 1980; Tsuruhara *et al.*, 1977).

ctivity of testicular acid phosphatase and hyaluronidase enzymes have been gested as the enzyme products of hormone induced protein synthesis during uration of germinal epithelium (Steinberger and Steinberger, 1975; Prasad and ıyan, 1986, 1987). The increased levels of gonadotropin and testosterone have sed an increase in the activity of both the enzymes, though the activity of acid sphatase is not significantly different from the control values at 60 days of age.

he increase in testes weight and function of seminiferous tubules as reflected by increased activity of the enzymes are obviously the consequence of naloxone ıulated increase in LH, FSH and testosterone levels which stimulated the yme activity and macromolecular synthesis. Gonadotropin and testosterone ıulated the activity of hyaluronidase and acid phosphatase enzymes in testes les and Turkington, 1971; Prasad and Vijayan, 1986, 1987). However, a ıction in both hyaluronidase and acid phosphatase activity at 90 days of age, ite of the increased gonadotropin and testosterone levels, were observed. The sible mechanism underlying this is not clear and require further studies.

pioid peptides are known to have a variety of other effects. Blockade of opioid ptors by naloxone could cause changes in thyroid function also. There is ence to suggest that thyroid status influences gonadal sensitivity to adotropin (Hadley, 1984). Since thyroid stimulating hormone (TSH) release *se* was not measured in these experiments changes in thyroid status of these rats onadotropin and testosterone levels could play only a minor role in the effects aloxone. Measurement of TSH levels could reveal the role if any.

he data presented here indicates that EOP exert some regulatory role in the elopment of reproductive function in the adult male rat *via* controlling the hesis and secretion of gonadotropin and testosterone. The increase in adotropin and testosterone levels could possibly be due to decrease in negative back by testosterone at hypothalamic and/or pituitary level. Blockade of opioid ptors in neonatal rats by chronic treatment with naloxone disturb the siological levels of gonadotropin in adult rats.

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